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Investigation of miR-335-5p and Its Target Gene C1QA Associated with the Complement System in Conversion from Clinically Isolated Syndrome to Multiple Sclerosis

Ayşegül TÜRK[1,2], Cem İsmail KÜÇÜKALİ[3], Tuğba KÖSE[4], Zerrin KARAASLAN[3], Murat KÜRTÜNCÜ[5]
Esin BAYRALI ÜLKER[1,2], Deryanaz BİLLUR[1,2], Özlem TİMİRCİ KAHRAMAN[1]

[1]Department of Molecular Medicine, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Türkiye
[2]Department of Molecular Medicine, Institute of Graduate Studies in Health Sciences, Istanbul University, Istanbul, Türkiye
[3]Department of Neuroscience, Aziz Sancar Institute of Experimental Medicine, Istanbul University, Istanbul, Türkiye
[4]Department of Genetics and Bioengineering, Faculty of Engineering and Natural Sciences, Istanbul Bilgi University, Istanbul, Türkiye
[5]Department of Neurology, Faculty of Medicine, Istanbul University, Istanbul, Türkiye

ABSTRACT

Introduction: Multiple sclerosis (MS), an autoimmune disease, attacks the central nervous system, causing inflammation and damage. Diagnosed in four forms, many clinically isolated syndrome (CIS) patients progress to relapsing-remitting MS (RRMS). C1QA, a molecule linked to MS, might be a treatment target due to its abnormal activity in the disease. This study investigated mir-335-5p and its targeting C1QA expression as potential biomarkers for disease progression. This relationship was also evaluated from an epigenetic perspective between CIS, RRMS and control groups.

Methods: Peripheral blood mononuclear cells (PBMCs) were isolated from 18 CIS, 32 RRMS, and 16 control blood samples. RNA isolation and Quantitative Real-Time PCR (qRT-PCR) were performed to detect the expression levels of C1QA and miR-335-5p, while C1QA protein levels were determined using ELISA.

Results: The data revealed significant increases in both C1QA gene expression (p=0.0291) and miR-335-5p expression (p=0.0196) in CIS patients compared to controls. Although increased expression levels were observed for both parameters in RRMS patients versus controls, they did not reach statistical significance. Patients with RRMS showed lower levels of C1QA and miR-335-5p compared to those with CIS. Notably, the decrease in miR-335-5p was statistically significant (p=0.0442). No significant difference in C1QA protein levels was observed among the groups (p >0.05).

Conclusion: miR-335-5p and C1QA emerge as potential biomarkers for MS progression, exhibiting significant upregulation in CIS compared to controls. miR-335-5p also shows significant downregulation in RRMS compared to CIS. These findings support the potential of miR-335-5p for distinguishing and understanding the progression of both CIS and RRMS.

Keywords: C1QA, clinically isolated syndrome, expression, miR-335-5p, relapsing-remitting multiple sclerosis

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INTRODUCTION

Multiple sclerosis (MS) is a chronic autoimmune disease characterized by inflammation and demyelination in the central nervous system (CNS) (1). Globally, 2.9 million people are living with MS, with 58,401 individuals in Türkiye affected. Most MS cases are diagnosed between the ages of 20 and 50, and women are three times more likely to be diagnosed with MS than men (2,3). Multiple sclerosis presents with a wide range of symptoms that vary significantly between individuals, making it a major cause of neurological disability in young adults. The mechanisms underlying this variability in disease progression remain largely unknown, and there is currently no cure for MS (4). It is characterized by repeated episodes of neurological symptoms, often beginning with a single episode known as clinically isolated syndrome (CIS). Multiple sclerosis is then divided into four groups based on its severity: relapsing-remitting MS (RRMS), primary progressive MS (PPMS), and secondary progressive MS (SPMS). Around 85–90% of patients are diagnosed with RRMS (5,6).

The complement system, a crucial component of the innate immune system, consists of approximately 50 proteins that circulate in the bloodstream or are bound to cell surfaces. It is designed to rapidly detect and respond to attacks from external pathogens (7,8). The

Correspondence Address: Özlem Timirci Kahraman, Department of Molecular Medicine, Aziz Sancar Institute of Experimental Medicine, Istanbul University, 34093 Çapa, Istanbul, Türkiye • **E-mail:** ozlemtk@istanbul.edu.tr
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Highlights

- **C1QA, miR-335-5p increase in CIS may predict early MS progression.**
- **C1QA, miR-335-5p higher in RRMS vs. control but lower in CIS.**
- **miR-335-5p is upregulated in CIS and downregulated in RRMS compared to controls.**
- **miR-335-5p may be a candidate biomarker for distinguishing early MS.**

complement system functions through three different pathways: the classical, alternative, and lectin pathways. In the classical pathway, the C1 complement protein complex serves as the initial responder, composed of C1q, C1r, and C1s (9). Pathological and serological studies in MS patients, along with functional studies in the Experimental Autoimmune Encephalomyelitis (EAE) animal model, have long supported the hypothesis that the complement system plays a role in MS pathology (1,10). Complement activation has been demonstrated in both acute and chronic MS lesions, with the presence of C1q staining suggesting the significance of the classical pathway in complement activation (11). The discovery of expression early classical complement pathway molecules in neuronal synapses or neurotoxic glia has led to new hypotheses suggesting that dysregulation of the complement system may contribute to neurodegeneration in MS (12).

MicroRNAs (miRNAs) show promise as potential biomarkers for diagnosis and monitoring disease progression in various conditions, including MS (13). Studies analyzing miRNA expression in various body fluids and tissues from MS patients, including peripheral blood, plasma, serum, different peripheral cell types, and brain lesions, have revealed irregular miRNA patterns compared to healthy controls (14,15). miR-335-5p is among the miRNAs with altered expression in MS (14,16,17), and C1QA emerges as a potential target for miR-335-5p (18).

No research has yet investigated the relationship between miR-335-5p and C1QA in MS patients. Furthermore, no studies have explored this miRNA-protein correlation as potential biomarkers to distinguish between CIS and RRMS after the initial attack.

In this study, the levels of miR-335-5p and C1QA gene and protein expression were examined in serum and PBMCs from individuals with CIS, RRMS, and healthy controls. Our aim was to evaluate their potential as biomarkers, both in assessing the risk of MS compared to healthy individuals and in distinguishing differences between CIS and RRMS patients after the initial attack.

METHODS

Participants

This study enrolled two patient groups from the Demyelinating Diseases Outpatient Clinic of the Department of Neurology, Istanbul Faculty of Medicine, Istanbul University. All patients met the McDonald criteria (19) and hadn't received any immunosuppressive or immunomodulatory treatment in the past two months. The first group included 18 patients with CIS, while the second group comprised 32 patients with RRMS. A third group of 16 age– and gender-matched healthy volunteers served as controls (Table 1).

The study included adult males and females aged 18 and above. Participants were excluded if they were pregnant or menopausal. All participants were informed about the research purpose, scope, and methodology through an Informed Consent Form. Demographic information, including age, sex, alcohol consumption, smoking habits, and family history, was then recorded. This study was reviewed and approved by the Ethics Committee of the Istanbul Faculty of Medicine at its meeting number 20 on November 5, 2021.

Peripheral Blood Mononuclear Cell Isolation

Uncoagulated blood samples were diluted with PBS in a 1:2 ratio and mixed gently. The 1:2 dilution of blood and PBS was then added to the Ficoll at a 1:3 ratio. It was centrifuged at 3000 rpm for 20 minutes at 20°C, with a brake setting of 3. After centrifugation, the PBMC layer was collected and transferred to a new Falcon tube. PBMCs were washed twice with PBS using a 1:1 ratio, centrifuged at 1800 rpm for 10 minutes at 4°C with a brake setting of 9 each time. Subsequently, 1 ml of FBS was added to the Falcon tube, followed by pipetting. Cell counting was performed using a Thoma Chamber under a fluorescent microscope. Each vial containing 10 million cells was then mixed with 500 µL of a 20% DMSO/FBS mixture and stored at -80°C for freezing.

Analysis of C1QA and miR-335-5p Expression

Total RNA and miRNA isolation from PBMCs was performed following the protocol of The NucleoSpin miRNA Isolation Kit (Macherey-Nagel, Düren, Germany). Purity and concentration of both total RNA and miRNA samples were measured using a NanoDrop spectrophotometer. RNA concentrations were standardized to 10 ng for 1 µL at an optical density of 260 nm. Isolated total RNA was reverse-transcribed to cDNA using the SCRIPT cDNA Synthesis Kit (Jena Bioscience, Germany), while the miRNA samples were reverse-transcribed using the Single miRNA qPCR kit (A. B. T. Laboratory Industry, USA). Quantitative Real-Time PCR (qRT-PCR) was performed using the Jena qPCR Probes Master Mix kit for total RNA samples and the Single miRNA qPCR kit for miRNA (Jena Bioscience, Germany). β-Actin and U6 served as housekeeping genes for qRT-PCR data analysis.

ELISA

C1QA levels in serum samples were determined using the ABclonal Human C1QA Sandwich ELISA kit protocol (ABclonal Biotechnology,

Table 1. Demographic characteristics of the study groups

	CIS	RRMS	Control	p^1	p^2	p^3
Sex (female/male)	10/8	18/14	8/8	1.000	1.000	1.000
Age (X ± SD)	39.61±12.08	29.41±9.43	32.43±9.09	0.050	0.278	**0.003***
Smoking status (yes/no)	9/9	9/23	4/12	0.284	1.000	0.139
Alcohol use (Yes/No)	3/15	7/25	6/10	0.242	0.285	0.730

n: number of subjects; intergroup differences were examined using the chi-square test (X^2) and pairwise independent sample t-tests; the values in the table are presented as X ± SD (X: mean; SD: standard deviation); *: $p<0.05$; p^1: CIS-control; p^2: RRMS control; p^3: CIS-RRMS.

USA). Following the addition of the stop solution, the optical density of each well was measured spectrophotometrically at 450 nm using a Multiscan Spectrum microplate reader within 5 minutes.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9 software, with a significance level set at p <0.05. Data normality was assessed using the Shapiro-Wilk test. Non-normally distributed data for patients and controls were compared using the Fischer's Exact test and Mann-Whitney U test, respectively, to identify significant differences. Receiver operating characteristic (ROC) analysis was performed to assess group-specific outcomes. Normally, distributed protein levels were compared between groups using the Shapiro-Wilk test (t-test). Spearman correlation was used for correlation analysis.

RESULTS

Figure 1 presents the relative changes in C1QA gene expression in PBMCs isolated from control and patient groups, as measured by qRT-PCR. Clinically isolated syndrome group exhibited a significant 2.33-fold upregulation of C1QA (p=0.0291). The RRMS group exhibited a 1.35-fold increase in C1QA expression levels compared to controls, but the difference was not statistically significant (p=0.1072). Comparing the disease groups, RRMS patients showed a 0.58-fold decrease in C1QA expression levels compared to the CIS group, but this difference was not statistically significant (p=0.1670).

Figure 2 depicts the relative changes in miR-335-5p expression levels among control, CIS, and RRMS groups, as determined by qRT-PCR analysis. Analyzing miR-335-5p expression, the CIS group showed a significant 4.11-fold increase compared to controls (p=0.0196). While the RRMS group exhibited a 1.42-fold increase compared to controls, this difference was not statistically significant (p=0.1960). Interestingly, between disease groups, a statistically significant 0.34-fold decrease in miR-335-5p expression was observed in RRMS compared to CIS (p=0.0442).

Table 2 presents the p-values from the Mann-Whitney U test, comparing C1QA and miR-335-5p expression levels between groups. To evaluate the diagnostic accuracy of these biomarkers in terms of sensitivity and specificity, we employed ROC analysis. The ROC curves for C1QA are illustrated in Figure 3, and those for miR-335-5p are shown in Figure 4. Table 2 further provides the area under the curve (AUC), p-values, and confidence intervals for both genes.

ROC curve analysis revealed statistically significant differences in both C1QA (p=0.029) and miR-335-5p (p=0.020) expression levels between the CIS and control groups. In the CIS-RRMS comparison, miR-335-5p expression maintained statistical significance (p=0.043).

Analysis of C1QA protein levels in serum samples from CIS, RRMS, and healthy control groups using the sandwich ELISA method revealed no significant differences between the groups (p >0.05). Figure 5 shows the C1QA levels for each group. To assess the relationship between C1QA and miR-335-5p, we performed a correlation analysis to determine the strength and direction of their association within the study.

Due to the non-parametric nature of the data, we employed Spearman correlation analysis. Table 3 presents the results, revealing no statistically significant correlations between C1QA mRNA and miR-335-5p, miR-335-5p and C1QA protein, or C1QA mRNA and C1QA protein in any of the pairwise group comparisons (CIS-control, RRMS-control, and CIS-RRMS; p >0.05).

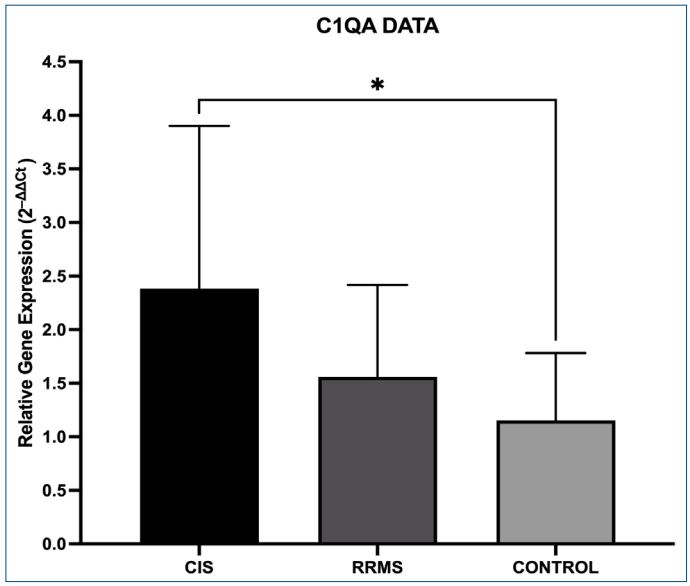

Figure 1. Comparison of C1QA gene expression levels in CIS, RRMS, and control groups (*p <0.05).

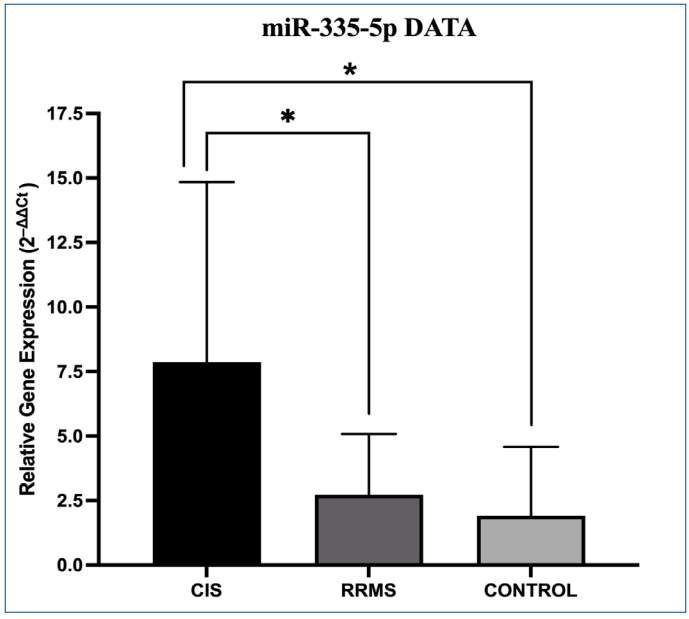

Figure 2. Analysis of miR-335-5p expression patterns in patients with CIS, RRMS, and healthy controls (*p <0.05).

Table 2. Comparative analysis of C1QA and miR-335-5p gene expression between study groups

Mann-Whitney U test		ROC analysis			
C1QA					
Group	p value	AUC	SE	p value	95% CI
CIS-control	**0.029***	0.73	0.09	**0.029***	0.54–0.93
RRMS-control	0.107	0.66	0.09	0.103	0.47–0.85
CIS-RRMS	0.167	6.64	0.10	0.159	0.43–0.86
miR-335-5p					
CIS-control	**0.019***	0.80	0.09	**0.020***	0.60–0.99
RRMS-control	0.196	0.65	0.11	0.187	0.42–0.85
CIS-RRMS	**0.044***	0.73	0.10	**0.043***	0.53–0.93

*p<0.05; statistically significant values are indicated bold; AUC: area under the curve; CIS: clinically isolated syndrome; miR: microRNA; ROC: receiver operating characteristic; RRMS: relapsing-remitting MS.

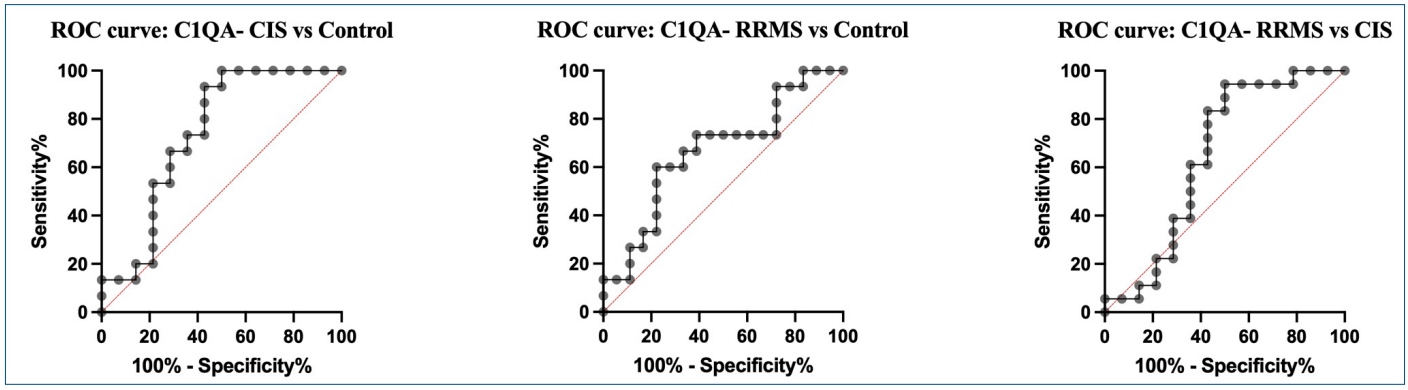

Figure 3. ROC curve analysis of C1QA as a biomarker for distinguishing control, CIS, and RRMS.

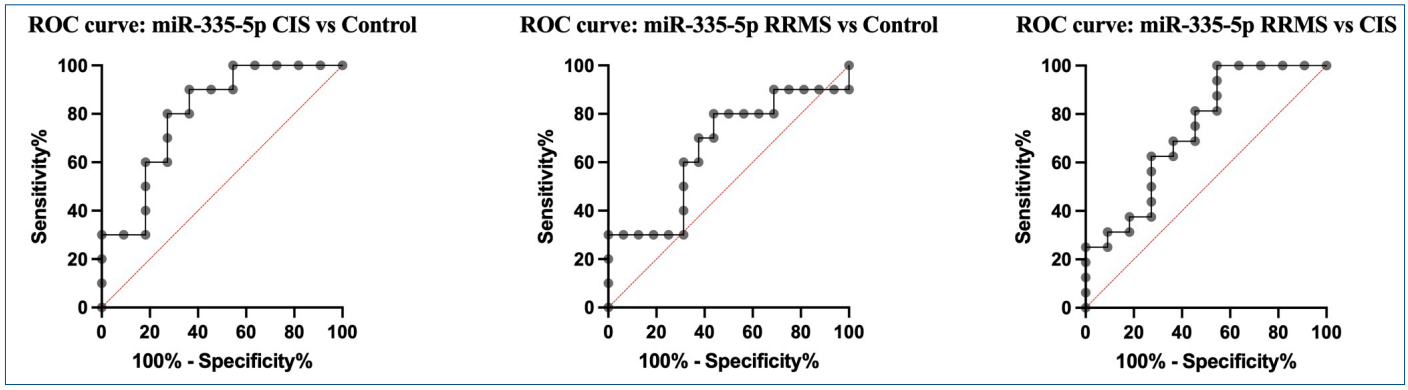

Figure 4. ROC curve analysis of miR-335-5p as a biomarker for distinguishing control, CIS, and RRMS.

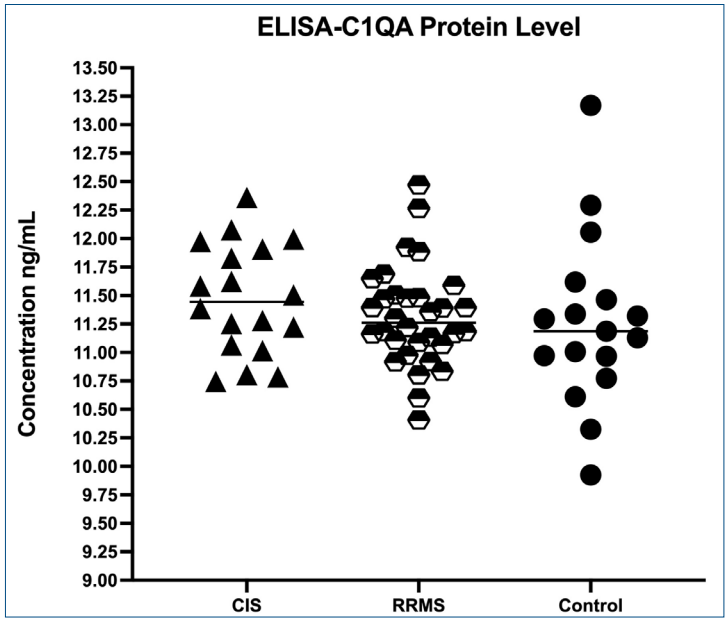

Figure 5. Comparison of serum C1QA concentration in CIS, RRMS, and control groups.

Table 3. Spearman correlation results

	C1QA mRNA/ miR-335-5p		miR-335-5p/ C1QA protein		C1QA mRNA/ C1QA protein	
	r	p	r	p	r	p
CIS-control	0.054	0.881	0.354	0.286	0.063	0.860
RRMS-control	-0.360	0.187	0.354	0.286	0.057	0.839
CIS-RRMS	-0.361	0.187	-0.075	0.791	0.057	0.840

*p<0.05; statistically significant values are indicated bold; r: correlation coefficient; C1QA: complement C1QA chain; miR: microRNA.

DISCUSSION

Multiple sclerosis is a chronic, immune-mediated neurological disorder characterized by inflammatory demyelination, oligodendrocyte depletion, and axonal loss, leading to plaques in the CNS (1). Diagnosis of MS relies on the established McDonald criteria (19). While white matter lesions on brain MRI remain crucial for differentiating MS, their appearance can mimic other neurological conditions, potentially leading to diagnostic errors. At this point, it is stated that immune system related genes and miRNA expression patterns can be associated with the diagnosis and prognosis of MS, and that these small molecules play an important role in both identifying the disease and predicting its course, enabling accurate classifications (20).

Complement C1q, a complex glycoprotein, mediates various immune regulatory functions that are considered important in the prevention of autoimmunity. While frequently observed in MS patients, the precise role and mechanisms by which C1q influences demyelination and synapse loss remain unclear (21). To assess whether enhanced complement expression and glial accumulation in the EAE model rendered synapses vulnerable to phagocytosis, Hammond et al. analyzed C1q protein and mRNA levels in the hippocampus via Western blot and qPCR. They found a 2.5-fold increase in C1q protein expression compared to control (22).

This study aimed to identify genes and miRNAs associated with the complement system in CIS, RRMS, and control groups. We sought to evaluate both the risk of MS compared to healthy individuals and the potential of these molecules as biomarkers for distinguishing between CIS and RRMS patients. Our selection of genes for analysis was informed by the proteomic data of Timirci Kahraman O et al., who utilized mass spectrometry to identify potential determinants of RRMS and CIS (6). Pathway analysis was conducted on genes with significantly altered protein expression in cerebrospinal fluid (CSF) samples from patients who remained with CIS and those who transitioned to RRMS, as identified by Liquid Chromatography Mass Spectrometry. Our analysis highlighted C1QA as a network-significant gene through STRING analysis. Furthermore, we investigated the potential of miR-335-5p, associated with C1QA, as a biomarker for MS patients, marking the first such exploration in the literature (6,18). This unique approach sets our study apart.

To assess whether complement activation markers correlate with disease activity, Håkansson et al. measured levels of C1q, C3, C3a, and sC5b-9 in the plasma and CSF of 41 patients (19 CIS, 22 RRMS) and compared them to 22 healthy controls. Notably, CSF-C1q levels were significantly higher ($p \leq 0.01$) in patients compared to the control group (1). In line with prior research, analysis of our study data revealed a statistically significant 2.3-fold increase in C1QA gene expression among CIS individuals compared to healthy controls ($p=0.029$) (1,23,24). Comparing RRMS patients to controls revealed a 1.35-fold increase in C1QA expression, although it wasn't statistically significant ($p=0.107$). Interestingly, within the RRMS group, C1QA expression showed a 0.58-fold decrease compared to CIS, but this difference also lacked statistical significance ($p=0.167$). We anticipate that increasing the sample size will alter this decrease in expression levels. The statistically significant increase in C1QA expression in the CIS group compared to the control group in this study validates the findings of the referenced proteomics study (6).

Gao et al. investigated the pathogenic role of C1q in RRMS by examining whether its expression levels differed in the peripheral blood of RRMS patients compared to healthy controls. Using a double antibody sandwich C1q-ELISA kit, they observed significantly higher C1q levels in RRMS patients (25). A Swedish thesis study compared C1q, C4, C3, fH, and C3a complement protein levels in plasma and CSF of individuals with MS, Guillan-Barré syndrome (GBS), other neurological diseases (OND), and controls. The study found no difference in plasma C1q levels between the groups (26). Håkansson et al. also reported that no significant difference was observed in terms of C1q expression levels between control and patient groups (CIS and RRMS) at plasma level (1). In the present study, we found no significant difference in C1QA protein levels between the control, CIS, and RRMS groups, as measured by ELISA.

A new study reveals that specific miRNAs circulating in the blood hold promise for both accurately classifying different stages of MS and identifying potential targets for innovative and powerful therapies (27).

Reviewing the literature on miRNA studies in the transition from CIS to RRMS reveals a focus on miR-181 c and miR-150. Interestingly, data from the miRTargetLink Human 2.0 database suggest a potential association between C1QA and miR-335-5p (18). It is known that many molecules, including miR-335-5p, can play critical regulatory roles in MS (28,29).

Muñoz-San Martín et al. examined the expression of 215 miRNAs in the CSF of individuals with RRMS, SPMS, spinal anesthesia, and other neurological diseases (migraine and dementia). Interestingly, they found that miR-335-5p, one of the analyzed miRNAs, exhibits higher expression levels in brain and spinal cord tissues (16). Juwik et al. reported down-regulated miR-335-5p expression in both lumbar motor and retinal neurons of EAE model mice, at both disease onset and peak stages (17). A 2021 doctoral thesis analyzed blood samples from 40 MS patients (encompassing a spectrum of MS subtypes) and 10 healthy controls for the expression levels of specific HLA-DRB1 and HLA-DQB1 alleles, as well as miR-204-5p and miR-335-5p, putative regulators of these genes. While no significant differences in gene alleles were detected between the control and MS groups, the patient group exhibited an up-regulation of miR-335-5p compared to the controls (30). Another study found that increased levels of miR-335-5p in both CIS and RRMS patients highlight its potential as a biomarker for early detection and disease monitoring (27). Additionally, miR-335 is known to play a role in the pathophysiology of MS. Among these miRNAs that show variability in MS lesions, it has been reported that the expression level of miR-335 is decreased in normal-appearing white matter (NAWM) (31).

The most remarkable and statistically significant ($p=0.0196$) finding of our study is the 4.11-fold increase in miR-335-5p levels in CIS patients compared to the controls While RRMS patients showed a 1.42-fold increase compared to healthy controls, it did not reach statistical significance ($p=0.196$). However, when comparing CIS and RRMS groups, we observed a statistically significant ($p=0.04$) 0.34-fold decrease in miR-335-5p expression in RRMS patients. This differential expression pattern suggests a potential role for miR-335-5p in the transition from CIS to RRMS. Although previous findings suggested potential interplay between C1QA and miR-335-5p, our correlation analyses failed to detect any statistically significant associations between these markers or with C1QA protein levels in any group comparisons (CIS-control, RRMS-control, and CIS-RRMS; all $p > 0.05$)

Despite numerous studies on miRNAs in MS, no single miRNA has gained widespread acceptance for diagnosis or treatment. While various studies have examined C1QA levels in MS patient samples, the present study breaks new ground by investigating the expression of miR-335-5p and its correlation with C1QA specifically in CIS and RRMS groups. This novel examination of these complement system-associated molecules, C1QA and miR-335-5p, across CIS, RRMS, and control groups represents a significant contribution to the field. Notably, our findings of increased C1QA expression in CIS patients compared

to controls align with Håkansson et al.'s 2020 study, further validating our approach (1). This analysis validating the elevated C1QA expression observed in the referenced proteomics study strengthens the evidence for its role in the context of our study (6). Though the observed decrease in C1QA expression among RRMS patients compared to CIS did not reach statistical significance, further investigation in a larger study group is warranted to confirm this trend and establish potential significance.

In conclusion, miRNAs hold immense promise as biomarkers in MS, and our findings further highlight this potential. Our study highlights the potential of C1QA and miR-335-5p as candidate biomarkers for MS. While both markers exhibited statistically significant differences in CIS compared to controls, miR-335-5p also showed significance in the CIS-RRMS comparison, suggesting its potential role in disease progression. The increased miR-335-5p levels in CIS group compared to controls suggest its significance in disease progression. The statistically significant rise in CIS is particularly exciting, indicating its potential role in early MS. Surprisingly, our study also presents a statistically significant decrease in miR-335-5p in RRMS compared to CIS. While limitations such as using different individual samples for CIS and RRMS, given the low sample size and long transition period, may contribute to this finding, it warrants further investigation. Ultimately, the potential of C1QA and miR-335-5p as biomarkers remains promising. To guide future studies and establish their validity as prognostic markers in MS, our unique results require validation through more extensive clinical trials with larger cohorts.

Ethics Committee Approval: This study was reviewed and approved by the Ethics Committee of the Istanbul Faculty of Medicine at its meeting number 20 on November 5, 2021.

Informed Consent: Informed consents were obtained from all patients or their legal guardians and controls, following provision of detailed information on the study examinations and tests.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept- AT, OTK; Design- AT, OTK; Supervision- OTK; Resource- OTK; Materials- MK; Data Collection and/or Processing- AT, CIK, TK, ZK, MK, EBU; Analysis and/or Interpretation- AT, OTK; Literature Search- AT, OTK, DB; Writing- AT, OTK, DB; Critical Reviews- OTK.

Conflict of Interest: The authors declared that there is no conflict of interest.

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