

# Treatment status influences circulating microRNA expression and their clinical and radiological associations in multiple sclerosis

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# Abstract

Circulating microRNAs (miRNAs) are promising biomarkers in multiple sclerosis (MS) due to their involvement in the regulation of multiple disease-related pathways. The aim of this study was to evaluate the impact of treatment status on the expression of a selected miRNAs panel and to investigate their associations with clinical and radiological variables in patients with MS (pwMS). Cross-sectional study was conducted in pwMS. Clinical disability was measured with Expanded Disability Status Scale (EDSS), cognitive function using the Processing Speed Test (PST), and brain volumetry using NeuroQuant®. Serum neurofilament light chain (sNfL) and glial fibrillary acidic protein (sGFAP) were quantified using Simoa®. MiRNAs were quantified by quantitative PCR following serum RNA extraction. Most analyzed miRNAs differed significantly between treated and untreated patients in both expression levels and variability. In treated patients, miR-9-5p was positively correlated with EDSS, whereas in untreated patients, miR-223-3p and miR-146a-5p were positively correlated with PST. Moreover, miR-9-5p was negatively associated, whereas miR-223-3p was positively associated with volumes of multiple brain regions. In addition, miR-126-3p discriminated between MS phenotypes. These findings support miR-9-5p, miR-223-3p, miR-146a-5p and miR-126-3p as candidate MS biomarkers and demonstrate that treatment status influences both their expression and their associations with clinical and radiological variables.

## INTRODUCTION

MicroRNAs (miRNAs) are endogenous small non-coding RNA molecules, typically 20–24 nucleotides in length, that play a crucial role in the post-transcriptional regulation of gene expression. They exert their regulatory effects by binding to complementary messenger RNA (mRNA) sequences, thereby inhibiting translation or promoting mRNA degradation<sup>1</sup>. Due to their remarkable stability in biological fluids and their involvement in numerous physiological and pathological processes, circulating miRNAs have emerged as promising minimally invasive biomarkers for a wide range of diseases<sup>2</sup>.

Accumulating evidence indicates that miRNAs regulate blood-brain barrier integrity, demyelination, remyelination, and neuroaxonal degeneration, suggesting a broad involvement in both the inflammatory and neurodegenerative aspects of multiple sclerosis (MS)<sup>3–8</sup>. Consistent with their role in MS pathogenesis, circulating miRNAs have emerged as candidate biomarkers for the disease<sup>9</sup>. Several studies have demonstrated altered miRNA expression profiles in patients with MS (pwMS) compared with healthy controls<sup>10</sup>, as well as differences according to disease activity (relapse versus remission)<sup>11</sup>, clinical phenotypes<sup>12</sup> and the therapeutic response to disease-modifying therapies (DMTs)<sup>13</sup>. However, further studies are needed to clarify whether treatment status influences circulating miRNA expression and their associations with clinical and radiological outcomes, which is essential for establishing their clinical utility as biomarkers in MS.

In previous studies, our group identified associations between a panel of circulating miRNAs and clinical disability, cognitive function, axonal damage, and brain atrophy in pwMS<sup>14–16</sup>, supporting their potential

role as biomarkers of MS. The present study aimed to validate these findings in a larger cohort of pwMS and to determine whether these miRNA associations differ according to treatment status.

## **MATERIALS AND METHODS**

### **1. Study Design**

Cross-sectional study in a cohort of pwMS attending the demyelinating disease unit at the Hospital Universitario de Getafe, Hospital Universitario Ramón y Cajal, and Hospital Universitario de Torrejón (Madrid, Spain).

We selected patients with a diagnosis of MS according to the 2017 revised McDonald criteria <sup>17</sup> and patients with radiologically isolated syndrome (RIS) according to the 2009 Okuda criteria <sup>18</sup>. Patients were classified as treated or untreated according to DMT exposure within the six months prior to blood sampling.

Demographic and clinical data, including sex, age at disease onset, and age at sampling were collected through a medical chart review during clinic visits. Clinical disability was measure with the Expanded Disability Status Scale (EDSS). Cognitive function was evaluated using the Processing Speed Test (PST). Raw values of PST were automatically transformed to Z-score that are adjusted for age and education.

All participants provided their consent to participate in the study. The study was conducted in accordance with the Helsinki Declaration and received approved by the ethical committee of the Hospital Universitario Fundación Jiménez Díaz (Madrid, Spain).

### **1. microRNAs Isolation and Analysis**

Peripheral blood samples were collected from all participants in additive-free red-top vacutainer tubes (BD Vacutainer®, Franklin Lakes, NJ, USA). Serum was separated by centrifugation at 920 x g for 15 min at room temperature and stored at -80°C until RNA extraction. For RNA isolation, the frozen serum was thawed at room temperature. To remove cryoprecipitates, 300 µL of thawed serum was centrifuged at 3000 x g for 5 min at 4°C, and 200 µL of the resulting supernatant was transferred to a new tube. Circulating RNA was extracted using the miRNeasy Serum/Plasma Advanced Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA isolation efficiency was monitored by adding the UniSp2, UniSp4, and UniSp5 Spike-in Mix (RNA Spike-in Kit for RT, Qiagen®, Germantown, MD, USA) during the extraction procedure. The total isolated RNA was reverse transcribed using the miRCURY LNA RT Kit (Qiagen®, Hilden, Germany), according to the manufacturer's instructions, to generate universal cDNA for all miRNAs present in the samples. The UniSp6 RNA Spike-in Mix (Qiagen®, Hilden, Germany) was included to each reaction to assess cDNA synthesis efficiency. Reverse transcription reaction was performed using the Veriti™ Thermal Cycle (Applied Biosystem™, Waltham, MA, USA) and the resulting cDNA were stored at -80°C until further analysis.

To evaluate RNA isolation efficiency and miRNA quality, we used the miRCURY LNA miRNA QC PCR Panel (Qiagen®, Hilden, Germany). This panel includes matching locked nucleic acid (LNA) PCR assays for the detection of the RNA spike-ins (UniSp2, UniSp4, and UniSp5) to monitor RNA isolation; UniSp6 and cel-miR-39-3p (not added in our experiments) to monitor cDNA synthesis; the UniSp3 IPC (inter-plate calibrator) to verify qPCR success; four potential endogenous miRNAs: miR-103-3p, miR-191-5p, miR-30c-5p, and miR-124-3p, as well as miR-451a and miR-23a-3p, which serve as hemolysis markers.

miRNA-specific quantification was carried out using the miRCURY LNA SYBR green kit (Qiagen®, Hilden, Germany) according to the manufacturer's instructions, on a LightCycler 96 instrument (Roche Applied Science, Basel, Switzerland). Only samples that demonstrated successful miRNAs extraction and reverse transcription, as well as the absence of hemolysis, as assessed by the miRCURY LNA miRNA QC PCR Panel, were included in the miRCURY LNA miRNA Custom PCR Panel analysis. A total of eight miRNAs previously associated with clinical disability, cognitive function, and brain atrophy in previous studies<sup>14–16</sup> (miR-9-5p, miR-29b-3p, miR-34a-5p, miR-126-3p, miR-138-5p, miR-146a-5p, miR-200c-3p, and miR-223-3p), were included in the custom PCR panels, together two potential endogenous miRNAs (miR-191-5p and miR-30c-5p) and the spike-ins UniSp6 and UniSp3.

All samples were run in duplicate. Only samples in which both technical replicates showed positive amplification were considered for downstream analysis. Normalized cycle quantification (Cq) values were obtained by subtracting the mean Cq of the endogenous controls (miR-191-5p and miR-30c-5p) from the mean of each miRNA.

## **1. Serum Neurofilament Light Chain and Glial Fibrillary Acidic Protein Analysis**

Serum neurofilament light chain (sNfL) and serum glial fibrillary acidic protein (sGFAP) levels were measured using the SR-X instrument (Quanterix, Lexington, MA, USA) with the single molecule array (Simoa) NF-light Advantage Kit and GFAP Advantage Kit (Quanterix, Billerica, MA, USA), respectively, according to the manufacturer's instructions. Raw sNfL values were subsequently transformed into age-adjusted Z-scores.

## **1. Brain Volume Analysis**

MRI images were acquired in accordance with the MAGNIMS recommendations for brain MRI in MS<sup>19</sup>, using scanners with a magnetic field strength of at least 1.5T and a maximum slice thickness of 3 mm without a gap. The imaging protocol included axial pre- and post-gadolinium T1-weighted sequences, axial proton density and/or T2-weighted, and axial and sagittal T2-fluid-attenuated inversion recovery.

A sagittal isovolumetric T1-weighted spoiled gradient recalled (3D-SPGR) sequence was acquired using the following parameters: TR = 8.5 ms; TE = 3.2 ms; TI = 700 ms, flip angle (FA) = 12°, bandwidth = 31.25 kHz, and the highest voxel size of 1 x 1 x 1 mm<sup>3</sup> for volumetric analysis. Volumetric measurements of the whole brain, cortical gray matter, cerebral white matter, cerebellum, thalamus, caudate, pallidum and

putamen were obtained using the automated mbrain Brain Volumetry module® and NeuroQuant® MS software.

## 1. Statistics

Statistical analyses were performed using the Statistical Package for Social Sciences, version 21.0 (IBM Corp., Armonk, NY, USA). Continuous variables were presented as mean  $\pm$  standard deviation for normally distributed data and as median and interquartile range for non-normally distributed data. Categorical variables were reported as percentages. Normality was assessed using the Shapiro-Wilk test. Correlations between miRNAs expression levels and clinical parameters and fluid biomarkers, including EDSS score, cognitive status, sNfL levels, sGFAP levels, and MRI findings, were evaluated using Pearson's correlation coefficient when both variables were normally distributed and Spearman's rank correlation coefficient for non-normally distributed data. Differences in miRNA levels across clinical MS subtypes were compared using the Mann-Whitney U test. Linear regression analyses were performed to evaluate whether significant associations between miRNA expression and continuous clinical or imaging variables remained after adjustment for age, when appropriate. Receiver operating characteristic (ROC) curve analysis was performed to assess the discriminative ability of selected miRNAs between different MS phenotypes, and the area under the curve (AUC) was calculated. For comparisons between treated and untreated patients, differences in the miRNA level were assessed using the Mann-Whitney U test for non-normally distributed variables and Student's t-test for normally distributed variables. Homogeneity of variances between groups was evaluated using Levene's test. Binary logistic regression analysis was performed to evaluate whether the association between miRNA expression and MS phenotype remained significant after adjustment for treatment status. Statistical significance was defined as a two-sided p-value  $< 0.05$ .

## RESULTS

We recruited 81 pwMS (55 females) including 65 relapsing remitting multiple sclerosis (RRMS), 6 secondary progressive multiple sclerosis (SPMS), 5 primary progressive multiple sclerosis (PPMS) and 5 RIS. Baseline demographic and clinical characteristics of the study cohort are summarized in Table 1.

Table 1  
Demographic and clinical data.

| <b>Sex (n (F/M))</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | <b>All patients</b> | <b>RIS</b>      | <b>RRMS</b>        | <b>SPMS</b>         | <b>PPMS</b>      |
|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------|-----------------|--------------------|---------------------|------------------|
|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | <b>81 (55/26)</b>   | <b>5 (4/1)</b>  | <b>65 (44/21)</b>  | <b>6 (4/2)</b>      | <b>5 (3/2)</b>   |
| <b>Untreated (n (%))</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | 36 (44.4)           | 5 (100)         | 24 (36.9)          | 3 (50)              | 4 (80)           |
| <b>High Efficacy Treatments (n (%))</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | 9 (11.2)            | -               | 7 (10.8)           | 1 (16.7)            | 1 (20)           |
| <b>Moderate Efficacy Treatments (n (%))</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | 36 (44.4)           | -               | 34 (52.3)          | 2 (33.3)            | -                |
| <b>Age at MS onset (years, mean <math>\pm</math> SD)</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | 33.5 $\pm$ 10.04    | 44.2 $\pm$ 4.6  | 32.2 $\pm$ 9.3     | 33.9 $\pm$ 13.9     | 39.4 $\pm$ 12.6  |
| <b>Age at sampling (years, mean <math>\pm</math> SD)</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | 42.01 $\pm$ 11.1    | 45.2 $\pm$ 3.3  | 40.9 $\pm$ 11.5    | 50.5 $\pm$ 8.1      | 43.0 $\pm$ 9.7   |
| <b>EDSS (median (P25, P75))</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | 2.0 (1.0, 3.0)      | 1.0 (0.5, 2.5)  | 2.0 (1.0, 2.5)     | 4.5 (3.5, 6.0)      | 3.0 (2.25, 4.25) |
| <b>PST (mean <math>\pm</math> SD)</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | 48.5 $\pm$ 11.7     | 50.6 $\pm$ 9.6  | 49.1 $\pm$ 12.6    | 42.5 $\pm$ 9.3      | 46.8 $\pm$ 8.3   |
| <b>sNfL (median (P25, P75))</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             | 8.02 (5.8, 13.5)    | 9.36 (4.4, -)   | 7.515 (5.6, 13.3)  | 115.9 (5.2, 19.7)   | 9.7 (8.1, 10.7)  |
| <b>sGFAP (median (P25, P75))</b>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | 92.6 (64.8, 136.4)  | 152.7 (79.1, -) | 89.6 (64.2, 134.6) | 165.8 (68.1, 250.5) | -                |
| n: Number of patients; F: Female; M: Male; MS: Multiple sclerosis; SD: Standard deviation; EDSS: Expanded disability status scale; PST: Processing speed test; sNfL: Serum neurofilament light chain; sGFAP: Serum glial fibrillary acidic protein; P25: Percentile 25; P75: Percentile 75; RIS: Radiologically isolated syndrome; RRMS: relapsing-remitting multiple sclerosis; SPMS: secondary progressive multiple sclerosis; PPMS: primary progressive multiple sclerosis. High Efficacy Treatments: Fingolimod, Cladribine and Ocrelizumab. Moderate Efficacy Treatments: Teriflunomide, Glatiramer acetate, Dimethyl fumarate and Interferon beta-1a. |                     |                 |                    |                     |                  |

## Differences in miRNAs expression according to treatment status

Significant differences in miRNA expression were detected between treated and untreated patients. Levels of miR-126-3p ( $p < 0.001$ ), miR-138-5p ( $p < 0.001$ ), miR-146a-5p ( $p < 0.0001$ ), miR-29b-3p ( $p = 0.036$ ) and miR-34a-5p ( $p = 0.001$ ) differed significantly between the two groups.

Since treated and untreated patients differed significantly in age ( $p = 0.036$ ), separate linear regression models were fitted for each miRNA, including treatment status as the independent variable and age as a covariate. The age-adjusted associations between treatment status and miRNA expression are presented in Fig. 1.

Assessment of variance using Levene's test showed that the variability of miRNA concentrations differed significantly between treated and untreated patients for six of the eight miRNAs analyzed (miR-138-5p,

miR-146a-5p, miR-200c-3p, miR-223-3p, miR-29b-3p, and miR-34a-5p; all  $p < 0.05$ ). For these miRNAs, treated patients generally exhibited lower variability than untreated patients. No significant differences in variance were detected for miR-126-3p ( $p = 0.140$ ) and miR-9-5p ( $p = 0.079$ ).

## **Associations between circulating miRNAs and clinical variables, sNfL, and sGFAP according to treatment status**

In treated patients, miR-9-5p showed a positive correlation with EDSS scores (Fig. 2A). In untreated patients, miR-146a-5p and miR-223-3p were positively correlated with PST values (Fig. 2B and 2C).

## **Associations between circulating miRNAs and brain volumetric measures according to treatment status**

In the treated group, miR-223-3p showed positive correlations with several brain volumetric measures, including whole brain ( $r = 0.502$ ;  $p = 0.006$ ), cerebral white matter, cortical gray matter, caudate, putamen, thalamus ( $r = 0.596$ ;  $p < 0.001$ ) and cerebellum volumes. In contrast, miR-9-5p was negatively associated with whole brain, cerebral white matter, putamen and thalamus volumes ( $r = -0.739$ ;  $p < 0.001$ ). Additionally, miR-200c-3p was negatively associated with pallidum volume ( $r = -0.533$ ;  $p = 0.005$ ) (Fig. 3).

No significant associations between circulating miRNAs and brain volumetric measures were observed in the untreated group.

## **Differences in miRNAs expression across MS phenotypes**

miR-126-3p levels differed significantly between RRMS and progressive forms, including PPMS and SPMS (PMS), as assessed by the Mann-Whitney U test ( $p = 0.0008$ ) (Fig. 4A). After adjustment for treatment status, phenotype remained independently associated with miR-126-3p expression ( $p = 0.033$ ). ROC curve analysis demonstrated a good discriminatory performance of miR-126-3p between RRMS and PMS, with an AUC of 0.8063 (95% CI, 0.6682–0.9443;  $p = 0.0012$ ) (Fig. 4B).

## **DISCUSSION**

The present study validates and extends our previous findings by demonstrating that the circulating miRNA panel previously identified by our group retains its potential as a minimally invasive biomarker panel for MS<sup>14–16</sup>. Furthermore, our findings demonstrate that treatment status significantly influences both circulating miRNA expression and their associations with clinical and MRI measures, indicating the importance of considering treatment status when evaluating circulating miRNAs as biomarkers in MS.

One of the main findings of this study was that DMTs significantly influences the expression profile of several circulating miRNAs included in our panel. After adjustment for age, treatment status remained

significantly associated with the expression of six of the eight miRNAs analyzed (miR-126-3p, miR-138-5p, miR-146a-5p, miR-200c-3p, miR-29b-3p, and miR-34a-5p), indicating that these differences cannot be explained simply by demographic differences between treated and untreated patients. Given that many of these miRNAs regulate pathways involved in immune responses, inflammation, and neurodegeneration<sup>20–24</sup>, it is biologically plausible that their expression is modulated by the immunomodulatory effects of DMTs. Our findings are consistent with previous studies reporting treatment-associated changes in circulating miRNA expression following DMT administration<sup>25,26</sup>.

Interestingly, treatment was associated not only with differences in the expression levels of several miRNAs but also with reduced variability in their circulating concentrations. This finding suggests that DMTs may contribute to stabilizing the expression of specific miRNAs by reducing inter-individual heterogeneity in immune and inflammatory responses.

One of the circulating miRNAs most strongly influenced by treatment status was miR-146a-5p, whose expression levels were significantly higher in treated than in untreated patients. This finding is biologically plausible, as miR-146a-5p is recognized as a key negative regulator of inflammatory responses, acting as a feedback mechanism that limits excessive activation of the NF- $\kappa$ B pathway and other innate immune signaling pathways<sup>6,22</sup>. In this context, the increased expression of miR-146a-5p observed in treated patients could reflect the immunomodulatory effects of DMTs, as several studies have demonstrated that these therapies attenuate NF- $\kappa$ B signaling and downregulate the expression of pro-inflammatory genes, thereby promoting a less inflammatory immune environment<sup>27,28</sup>.

Moreover, among untreated patients, higher miR-146a-5p concentrations were associated with better cognitive function, which is consistent with a recent study that reported reduced miR-146a-5p expression in pwMS with cognitive impairment, identifying this miRNA as one of the key regulators of the molecular networks underlying cognitive dysfunction<sup>29</sup>.

Another important finding of this study was the ability of miR-126-3p to discriminate between patients with RRMS and PMS, with higher concentrations observed in patients exhibiting a progressive disease phenotype, this association remained significant after adjustment for treatment status. This finding suggests that differences in miR-126-3p expression are more related to intrinsic characteristics of the clinical phenotype than to the effects of DMTs, reinforcing its potential value as a biomarker of disease progression. Consistent with this observation, in our previous study we found that higher circulating levels of miR-126-3p were associated with confirmed disease progression at 2 years<sup>16</sup>.

Although the role of miR-126-3p in MS pathophysiology has not been fully established, experimental evidence supports its potential involvement in neurodegenerative mechanisms. Kim et al.<sup>23</sup> demonstrated that miR-126 overexpression suppresses the IGF-1/PI3K/AKT signaling pathway through the expression regulation of IRS-1, PI3K p85 $\beta$ , and SPRED1, thereby reducing IGF-1-mediated neuroprotective signaling and increasing neuronal susceptibility to injury. Inversely, the inhibition of miR-126 partially restored this pathway and improved neuronal survival. Although this study was conducted

using a Parkinson's disease model, it could be suggested that similar mechanisms may also operate in PMS, a disease stage characterized by predominant neurodegeneration.

This hypothesis is particularly interesting considering that, although most studies have reported no significant differences in serum IGF-1 concentrations between pwMS and healthy controls<sup>30,31</sup>, reduced IGF-1 levels have been described in patients with PPMS and in those with higher EDSS scores. Moreover, lower serum IGF-1 concentrations have been associated with an increased risk of cognitive impairment<sup>30</sup>. Collectively, these findings support the involvement of the IGF-1 pathway in neurodegeneration and disease progression in MS and suggest that miR-126-3p may regulate this pathway. Nevertheless, functional studies in MS are required to clarify the role of miR-126-3p in neurodegeneration.

Another significant finding was the association of miR-223-3p with different indicators of brain integrity, depending on treatment status. In untreated patients, higher miR-223-3p expression was associated with better cognitive function, whereas in treated patients it positively correlated with several brain volumetric measures, including whole brain, cerebral white matter, cortical gray matter, thalamic, putamen, caudate, and cerebellar volumes. Together, these findings demonstrate a consistent association between higher circulating miR-223-3p expression and better preservation of brain structure and cognitive function.

These observations are biologically plausible, given that experimental studies on experimental autoimmune encephalomyelitis (EAE) have demonstrated that miR-223-3p exerts neuroprotective effects. They show that miR-223-3p overexpression in cortical neurons protected against neurite degeneration, whereas loss of this miRNA impaired neurite outgrowth<sup>20</sup>. Therefore, the association between higher circulating miR-223-3p levels and greater brain volumes observed in our study may reflect the activation of endogenous mechanisms aimed at limiting neuroaxonal damage.

miR-9-5p is a pro-inflammatory miRNA that has been implicated in MS pathogenesis by promoting macrophage polarization toward the M1 phenotype<sup>32</sup> and enhancing Th17 cell differentiation<sup>33</sup>. In the present study, higher miR-9-5p expression was associated with greater clinical disability and lower whole-brain, cerebral white matter, putamen, and thalamic volumes in treated patients. These findings are consistent with our previous observations<sup>16</sup>, in which elevated circulating miR-9-5p levels were associated with worse clinical progression at two years, further supporting its potential value as a biomarker in MS.

Notably, these associations were observed exclusively in treated patients, although no significant differences in miR-9-5p expression were found between treated and untreated patients. This finding suggests that miR-9-5p may serve as a biomarker of treatment response, instead than simply reflecting treatment exposure. In line with this interpretation, an experimental study identified miR-9-5p as one of the miRNAs whose expression varies during EAE progression and following treatment with glatiramer acetate, proposing it as a potential biomarker of disease progression and therapeutic response<sup>34</sup>. This hypothesis should be further investigated in longitudinal studies evaluating miR-9-5p dynamics in treatment responders and non-responders.

Overall, this study validates a panel of circulating miRNAs previously identified by our group as promising biomarkers for MS and demonstrates that treatment status influences both their expression and their associations with clinical and MRI parameters. These findings highlight the need for consider treatment status when interpreting circulating miRNAs as biomarkers in MS and further support their potential to provide complementary information for disease progression, neurodegeneration, and therapeutic response. However, the cross-sectional design and limited sample size in some clinical subgroups require that the results be interpreted with caution. Future longitudinal, multicenter studies are needed to confirm these observations and determine the prognostic value and clinical applicability of these circulating miRNAs as minimally invasive biomarkers of MS in clinical practice.

## Declarations

**Author contributions:** GPV, MIDM, SR and RAL performed the statistical analyses, discussed and interpreted the findings, and critically revised the manuscript. GPV, MIDM, ICP and RAL contributed to the design of the study. GPV, AAG, IPG and MAGM prepared and processed the samples. GPV prepared the original draft and performed the visualization. MIDM, ICP and RAL funding acquisition. LMV and NV prepared the samples for sNfL and sGFAP analyses and performed such analysis. EM, LCF, LMV, NV, YA and JDD resources. All authors have read and agreed to the published version of the manuscript.

### Additional information:

Guadalupe Pérez de Villar: nothing to disclose.

María Inmaculada Domínguez-Mozo: nothing to disclose.

Ignacio Casanova-Peno: has received payments for consulting services from Novartis and Merck, speaking honoraria from Merck, Novartis, Sanofi, and Neuraxpharm, support for attending meetings from Merck, and research grants from TEVA and Sanofi.

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Lucienne Costa-Frossard: reports compensation for consulting services and speaker honoraria from Biogen, Bristol Myers Squibb, Janssen, Merck-Serono, Novartis, Sanofi, Roche, and Teva.

Luisa María Villar: has served at scientific advisory boards, participated in meetings sponsored by, received speaking honoraria or travel funding or research grants from Roche, Sanofi, Merck, Biogen, Bristol Myers, and Novartis.

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**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients to publish this paper.

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## Figures

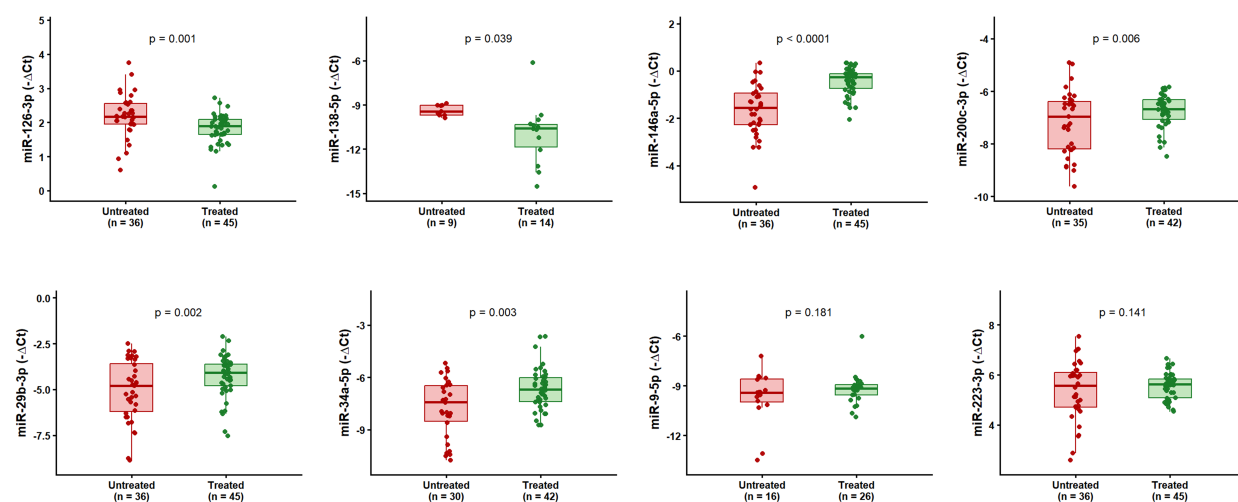


**Figure 1**

Differences in microRNAs expression levels between treated and untreated patients with multiple sclerosis using Mann-Whitney U test for non-normally distributed variables and Student's t-test for normally distributed variables. Data are presented as median and interquartile range (25<sup>th</sup>-75<sup>th</sup> percentile); dots represent individual participants. P values shown were adjusted for age. n, number of patients.

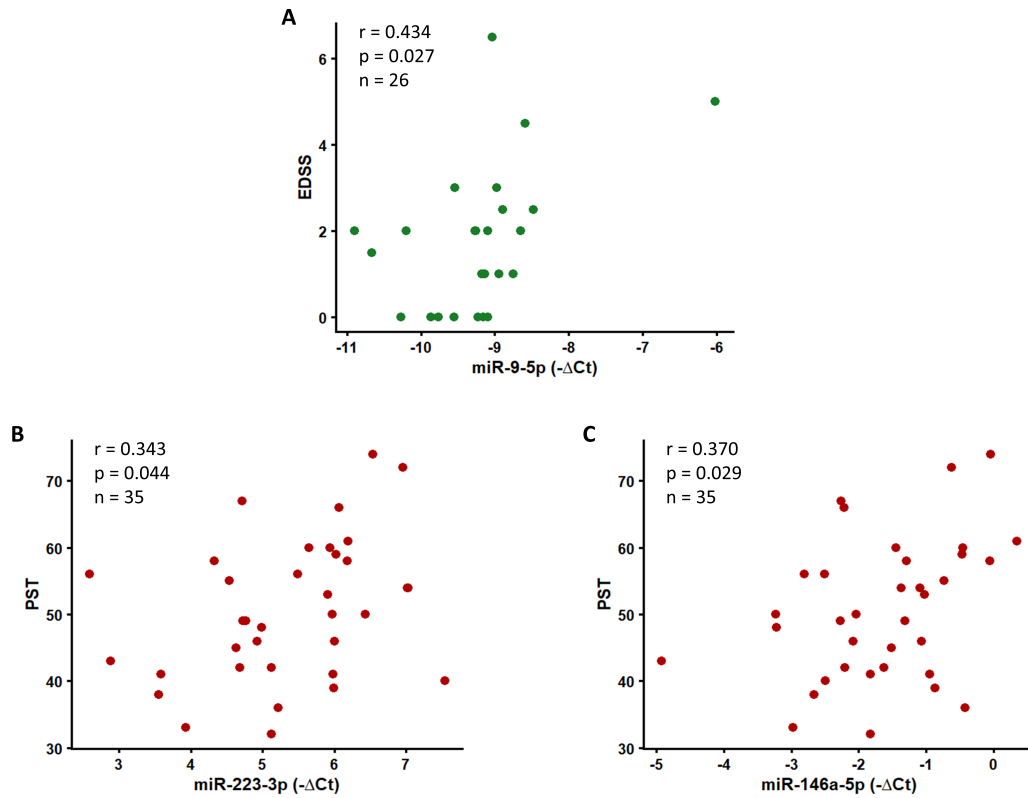


**Figure 2**

Correlations between microRNA expression levels and clinical variables in treated and untreated patients with multiple sclerosis. Green circles represent treated patients and red circle represent untreated patients. **(A)** Correlation between miR-9-5p expression levels and EDSS score in treated patients with multiple sclerosis using Spearman's correlation analyses. **(B)** Correlation between miR-223-3p expression levels and PST score in untreated patients with multiple sclerosis using Pearson's correlation analyses. **(C)** Correlation between miR-146a-5p expression levels and PST score in untreated patients with multiple sclerosis using Pearson's correlation analyses. EDSS, Expanded Disability Status Scale; PST, Processing Speed Test. n, number of patients.



**Figure 3**

Correlations between microRNA expression levels and brain volumes in treated patients with multiple sclerosis. Correlation coefficients correspond to Pearson's or Spearman's analyses according to data distribution. Samples sizes varied across miRNAs: 28 patients for miR-126-3p, miR-146a-5p, miR-223-3p, miR-29b-3p and miR-34a-5p; 26 for miR-200c-3p; 18 for miR-9-5p; and 10 for miR-138-5p. WBV, whole brain volume; WMV, white matter volume; CGMV, cortical grey matter volume. Volume in ml. Asterisks indicate statistical significance ( $p^* < 0.05$ ;  $p^{**} < 0.01$ ;  $p^{***} < 0.001$ ).

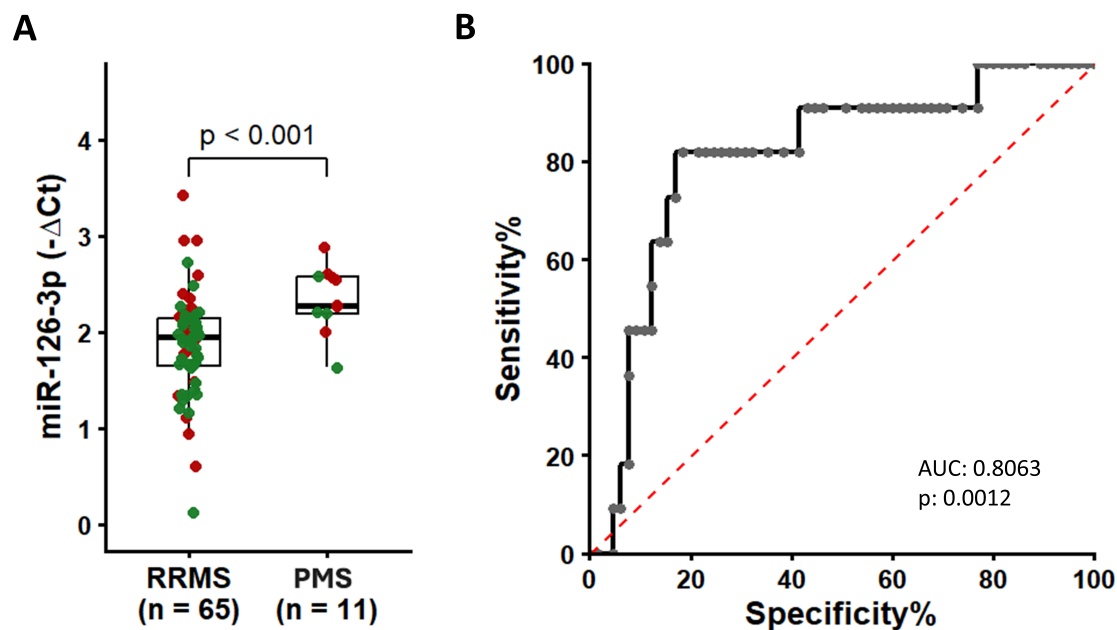


**Figure 4**

Differences in miR-126-3p expression across multiple sclerosis phenotypes. **(A)** Differences in miR-126-3p expression levels between multiple sclerosis phenotypes evaluated using the Mann-Whitney U test. The P values shown were obtained before adjustment for treatment status. Green circles represent treated patients and red circle represent untreated patients. **(B)** Receiver operating characteristic (ROC) curve evaluating the ability of miR-126-3p expression levels to discriminate relapsing-remitting multiple sclerosis (RRMS) from progressive multiple sclerosis (PMS). AUC, area under the curve. n, number of patients.