RESEARCH ARTICLE OPEN ACCESS

Micro-RNA Signature in CSF Before and After Autologous Hematopoietic Stem Cell Transplantation for Multiple Sclerosis

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Neurol Neuroimmunol Neuroinflamm 2025;12:e200345. doi:10.1212/NXI.0000000000200345

Abstract

Background and Objectives

MicroRNAs (miRNAs) are regulators of gene expression and have been reported to be dysregulated in people with multiple sclerosis (pwMS). Autologous hematopoietic stem cell transplantation (aHSCT) is an immune-ablative treatment intervention for pwMS. Currently, it is unknown if aHSCT affects expression levels of miRNAs in CSF. We explored the ability of circulating miRNA to discriminate between pwMS and healthy controls (HCs) and investigated whether these miRNAs were affected by treatment with aHSCT.

Methods

Using quantitative reverse transcription PCR, 87 miRNAs were analyzed in CSF samples of a discovery cohort (*baseline: 4 & HC: 4*). The top 22 miRNAs discriminating between pwMS and HCs were then analyzed in 187 CSF samples of a validation cohort (*pwMS: 50, HC: 32*). Samples, failing quality control or being follow-ups to baseline samples with quality control issues, were excluded from further analyses. The remaining 133 samples (*HC: 29, MS: baseline: 33, 1 year: 30, 2 years: 26, 3–5 years: 15*) were analyzed for expression of the top 22 miRNAs.

Results

Twelve miRNAs were dysregulated in pwMS compared with HC ($q < 0.05$). Associations with clinical and analytical parameters were observed in relation to all 12 miRNAs; however, a cluster of 4 miRNAs (miR-16-5p, miR-21-5p, miR-150-5p, and miR-146a-5p) with strong correlations ($r > 0.60$, $p < 0.001$) with multiple parameters was identified. Of the 12 miRNAs, 8 were differentially expressed in pwMS with gadolinium-enhancing lesions at baseline and 4 by prior disease-modifying treatment class ($p < 0.05$). These 4 miRNAs correlated strongly with each other, decreased after aHSCT, and remained low throughout the follow-up period ($p < 0.05$). Target and pathway analysis of these revealed association with biological processes affecting cytokine production, inflammatory response, and regulation of myelin maintenance.

Discussion

miRNAs are dysregulated in CSF from pwMS and particularly in patients with less effective treatments and/or higher inflammatory disease activity. A 4-miRNA signature with elevated expression of miR-16-5p, miR-21-5p, miR-150-5p, and miR-146a-5p was recurring in multiple analyses. After intervention with aHSCT, the expression levels approached the levels of the HCs, suggesting a potent treatment effect.

Introduction

Multiple sclerosis (MS) is an autoimmune disorder characterized by inflammation and neurodegeneration within the CNS. In its early stages, the disease is primarily driven by inflammatory processes, although neurodegenerative mechanisms become increasingly

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The Article Processing Charge was funded by The Swedish Research Council.

Glossary

AD = Alzheimer disease; **aHSCT** = autologous hematopoietic stem cell transplantation; **Cq** = cycle quantification; **DMT** = disease-modifying treatment; **EIDA** = evidence of inflammatory disease activity; **GO** = gene ontology; **HC** = healthy control; **miRNA** = microRNA; **mRNA** = messenger RNA; **MS** = multiple sclerosis; **pwMS** = people with multiple sclerosis; **qPCR** = quantitative PCR; **RRMS** = relapsing-remitting multiple sclerosis.

prominent with time.[1] There is an extensive literature on protein biomarkers in MS, but less attention has been given to exploring circulating nucleic acids in both blood and CSF as potential biomarkers for MS.

MicroRNAs (miRNAs) are short, noncoding, RNA molecules that regulate gene expression by binding to messenger RNAs (mRNAs). Through mRNA degradation or inhibition of translation, they impose a crucial influence on various biological processes and are implicated in a multitude of diseases and cellular functions, making them potential therapeutic targets and diagnostic markers in medical research.[2]

Dysregulation of miRNA expression in MS has seen increasing interest in recent years, and several miRNAs have been highlighted as potential biomarkers of MS in both blood and CSF.[3–5] A thorough understanding of miRNAs in people with MS people with multiple sclerosis (pwMS) and the possible influence of disease-modifying treatments (DMTs) is currently lacking.

Autologous hematopoietic stem cell transplantation (aHSCT) has been used as a treatment for MS since the mid-1990s.[6] This therapeutic approach involves immune system ablation using high-dose chemotherapy, followed by the reconstitution of a new immune system with the assistance of autologous hematopoietic stem cells.[7] Notably, a recent study indicated that 63% of pwMS treated with aHSCT in Sweden maintained a state of "no evidence of disease activity" for a decade following the procedure, obviating the need for additional treatments.[8] It is considered one of the most potent treatment interventions for MS and can serve as a model for intense immunosuppression.

This study had 2 aims: first to describe a CSF miRNA signature of pwMS and second to assess changes in this signature after treatment intervention with aHSCT.

Methods

Ethical Approvals

The research received approval from the Regional Ethical Board of Uppsala (Dnr 2008/182 and 2012/080/1). All participants provided informed written consent for their participation in the study in accordance with the Declaration of Helsinki.[9]

Participants

Patients scheduled for intervention with aHSCT using a cyclophosphamide-based conditioning regimen at Uppsala University Hospital between December 2011 and February 2020 were invited to take part in the study. All patients were diagnosed with relapsing-remitting multiple sclerosis (RRMS) according to the 2017 revision of the McDonald criteria.[10]

Based on prior DMTs, patients were divided into 3 groups: first-line, second-line, and treatment-naïve. First-line treatments included dimethyl fumarate, glatiramer acetate, interferon, and teriflunomide. Second-line treatments comprised fingolimod, rituximab, and natalizumab.

NEDA-2 was defined as the absence of new MRI events and clinical relapses. Patients who did not maintain NEDA-2 status during follow-up were said to have "evidence of inflammatory disease activity" (EIDA).

CSF Collection

The participants were asked to undergo lumbar puncture before aHSCT and subsequently at 1, 2, and 5 years of post-aHSCT. In cases where patients were unable to undergo lumbar puncture, such as during pregnancy, arrangements were made to perform the procedure at a later follow-up visit. Additional lumbar punctures were performed for some patients when relapse was suspected, to assess disease status. CSF samples from healthy controls (HCs) were collected at a single timepoint, and all CSF samples were processed in accordance with consensus guidelines.[11] Blood samples were collected simultaneously as part of routine health care.

Procedures

Autologous hematopoietic stem cells were mobilized through a single dose of 2 g/m^2 cyclophosphamide, followed by filgrastim at 5–10 μg/kg/d for 6–7 days, and harvested approximately 10 days after initiating the mobilization regimen. No ex vivo graft manipulation was performed. Patient conditioning involved a combination of cyclophosphamide and rabbit anti-thymocyte globulin (cyclophosphamide 200 mg/kg; rATG 6 mg/kg). Prophylaxis for fungal, viral, and bacterial infections was administered during neutropenia, with continued prophylaxis for herpes viruses and *Pneumocystis jiroveci* for a minimum of 3 months. The procedure has been described in detail elsewhere.[8]

Analysis of Circulating miRNA

Frozen CSF samples were sent to QIAGEN in Hilden, Germany, where they were prepared and analyzed according to the protocol described below.

Sample Preparation

CSF samples were thawed on ice and subjected to centrifugation at 3,000×*g* for 5 minutes in a microcentrifuge

maintained at 4°C. Subsequently, 200 µL aliquots per sample were transferred to FluidX tubes. To each sample, 60 µL of buffer RPL, containing 1 µg carrier RNA per 60 µL of buffer RPL and RNA spike-in template mixture, was added. The mixture was thoroughly mixed for 1 minute and then incubated for 7 minutes at room temperature. Following this, 20 µL of buffer RPP was added. Total RNA extraction from the samples was performed using the miRNeasy Serum/Plasma Advanced Kit, using the high-throughput bead-based protocol v.1 in an automated 96-well format (QIAGEN, Hilden, Germany). The purified total RNA was finally eluted in a volume of 50 µL.

miRNA Real-Time Quantitative PCR

Reverse transcription of 4 µL RNA was performed in 20 µL reactions using the miRCURY LNA RT Kit from QIAGEN. The resulting cDNA was diluted 50 times and assayed in 10 µL PCR reactions following the miRCURY LNA miRNA PCR protocol. Each miRNA was quantified once by quantitative PCR on the miRNA Ready-to-Use PCR, 205, using miRCURY LNA SYBR Green master mix. Negative controls, which excluded templates from the reverse transcription reaction, were performed and profiled alongside the samples. Amplification was conducted using the LightCycler 480 Real-Time PCR System (Roche, Basel, Switzerland) in 384-well plates. Amplification curves were analyzed using Roche LC software, both for determining the cycle quantification (Cq) through the second derivative method and for conducting melting curve analysis.

Data Analysis

Amplification efficiency was determined through algorithms resembling those found in LinReg software. Each assay underwent scrutiny for well-defined melting curves, ensuring that the melting temperature (Tm) aligned with known specifications for the assay. In addition, assays needed to be detected with a cycle quantification (Cq) of 5 cycles less than the negative control and with Cq < 37 to be considered for inclusion in the data analysis. Any data failing to meet these criteria were excluded from further analysis. The calculation of Cq was based on the second derivative. Normalization of all data was performed to minimize technical variability as well as enable accurate and biologically meaningful interpretations of miRNA expression patterns. Using NormFinder, the 5 most stable reference assays (*miR-27b-3p, miR-30c-5p, miR-16-5p, miR-100-5p, and miR-143-3p*) were selected as normalizers.

Statistical Analysis

Statistical analyses were performed in Qlucore Omics Explorer (version 3.9, Qlucore, Lund, Sweden).[12] Assessment of miRNAs of interest in the discovery cohort was performed with a significance level of $p < 0.05$. In the initial identification of miRNAs able to discriminate pwMS from HCs, $q < 0.05$ was considered statistically significant. After the miRNAs of interest were identified, a two-tailed p-value of 0.05 was considered significant for further analyses. Paired and correlation analyses were performed in GraphPad Prism version 9.5.0 for macOS (GraphPad Software.).[13] Student t tests with Bonferroni-Holm correction were used to assess differences between treatment groups. The unequal variances t test was used when assessing differences between pwMS with and without gadolinium-enhancing lesions. A Pearson correlation coefficient was used for assessing associations between the miRNAs, while a Spearman rank correlation coefficient was used for associations with clinical and analytical parameters.

Associations were classified according to the British Medical Journal guidelines.[14] Figures were built with Qlucore Omics Explorer or GraphPad Prism and graphically processed in Affinity Designer (v.1.10.5, Serif [Europe] LTD.).[15]

Data Availability

The data that support the findings of this study are available from the corresponding author, on reasonable request.

Results

The initial screening of miRNAs of interest was performed in a cohort of 4 pwMS and 4 HCs. Then, CSF samples obtained from 50 pwMS and 32 HCs were investigated. Following quality control assessments, 54 samples were found to be noncompliant and were excluded from subsequent analyses. In the final analysis, 133 samples from 33 pwMS (*baseline: 33; 1 year: 30; 2 years: 26; 3–5 years: 15*) and 29 HCs were included. The characteristics of the final cohort are presented in Table 1.

Characterization of CSF Samples

Routine analyses of blood and CSF were performed as part of standard health care (Table 2). Within the HC group, assessments of CSF cell counts and albumin levels indicated results within the expected normal ranges.

Discovery of miRNAs of Interest

The initial discovery analysis was performed on CSF samples from 4 pwMS and 4 age-matched and sex-matched HCs. The samples were analyzed for the expression of 87 CSF-relevant miRNAs (CSF exosome panel I, YAHS-124, QIAGEN). The top 22 miRNAs discriminating between pwMS and HC were selected based on their respective p-values. For normalization, 5 of the 22 miRNAs were chosen (miR-27b-3p, miR-30c-5p, miR-16-5p, miR-100-5p, and miR-143-3p).

Dysregulated miRNAs in CSF From pwMS

The final study cohort was comprised of 33 pwMS and 29 HCs. The Student two-tailed t test, adjusted for age and sex, revealed 12 miRNAs to be dysregulated in pwMS compared with HCs ($q < 0.05$) (Figure 1). Of these, 10 miRNAs were overexpressed in pwMS and 2 miRNAs were downregulated (eTable 1).

Correlation Matrix of Identified miRNAs

A correlation matrix was constructed to describe the association between the levels of the 12 dysregulated miRNAs in pwMS

Table 1 Demographics and Clinical Characteristics of the Cohort

	MS	Healthy control
N	**(n = 33)**	**(n = 29)**
Age at inclusion, mean (SD)	32 (7)	25 (8)
Sex, F/M (%women)	24/9 (73)	13/16 (45)
Expanded disability status scale, median (IQR)		
Baseline	3.5 (2.3–4.0)	NA
1 y	2.0 (1.0–3.5)	NA
2 y	1.5 (1.0–3.3)	NA
3–5 y	2.0 (1.5–3.5)	NA
Annual relapse rate, mean (SD)	1.6 (1.6)	NA
Disease duration (y), mean (SD)	6.3 (5.6)	NA
Number of previous treatments, median (IQR)	2 (1–3)	NA
All treatments		
Naïve	4	NA
1st-line	11	NA
2nd-line	18	NA
NEDA-3/EDA	24/9	NA

Abbreviation: MS = multiple sclerosis; IQR = interquartile range.

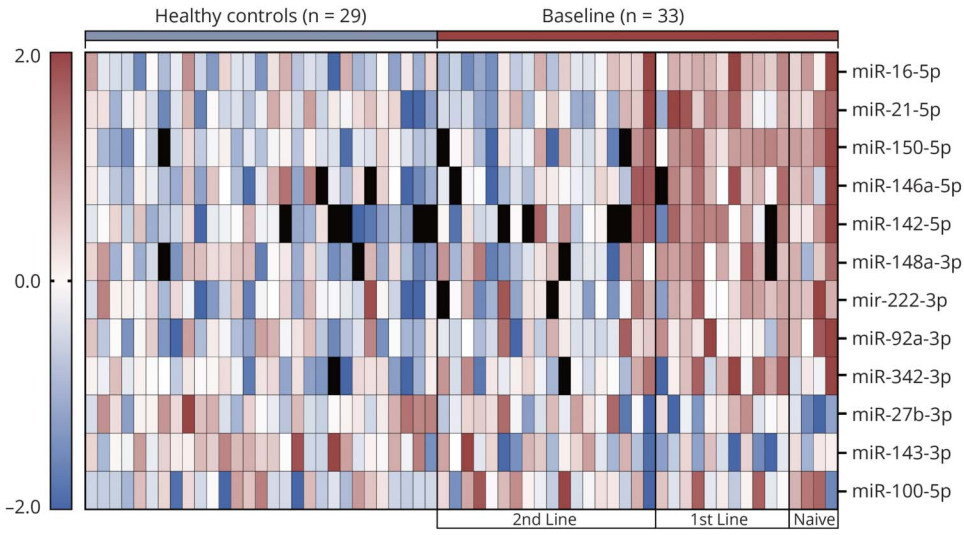
before aHSCT (eTable 2). A cluster of positive covariance was observed comprising 9 of the 12 miRNAs (miR-16-5p, miR-21-5p, miR-150-5p, miR-146a-5p, miR-142-5p, miR-148a-3p, miR-222-3p, miR-92a-3p, and miR-342-3p). Of these, 4 miRNAs (miR-16-5p, miR-21-5p, miR-150-5p, and miR-146a-5p) had strong associations with each other (Figure 2).

Table 2 Characterization of CSF Samples of the Cohort

Factor	Unit		HC (n = 29)	Baseline	n	1 y	n	2 y	n	3–5 y	n
Mononuclear cells	Million cells/L	Median (IQR)	<5	2.0 (1.0–5.3)	26	1.0 (0.0–2.0)	26	1.0 (0.0–2.0)	20	1.5. (0.3–3.0)	8
Polynuclear cells	Million cells/L	Median (IQR)	<1	0.0 (0.0–0.0)	26	0.0 (0.0–0.0)	26	0.0 (0.0–0.0)	20	0.0 (0.0–0.0)	8
Albumin	mg/L	Median (IQR)	<320	246 (160–310)	26	220 (150–290)	26	210 (170–300)	20	290 (200–350)	8
Albumin quota	CSF/S	Median (IQR)	<0.009	6.0 (3.7–8.0)	26	4.9 (3.3–6.7)	26	4.8 (3.7–8.4)	20	6.1 (4.9–8.8)	8
P-IgG	g/L	Median (IQR)	<14.5	9.2 (8.2–11)	26	9.2 (8.0–11)	26	9.3 (8.0–10)	20	9.4 (8.1–11)	8
CSF-IgG	mg/L	Median (IQR)	<45	46 (30–65)	26	30 (22–40)	26	28 (22–45)	20	34 (18–42)	8
IgG index	CSF/P	Median (IQR)	<0.6	0.8 (0.6–1.1)	26	0.6 (0.5–0.8)	26	0.6 (0.5–0.8)	20	0.5 (0.4–0.6)	8
IgG OCB	Yes/no		No	25/1	26	23/2	25	19/0	19	6/2	8
S-IgM	g/L	Median (IQR)	<2.1	0.8 (0.6–1.5)	24	0.8 (0.5–1.1)	22	0.7 (0.6–0.9)	19	0.8 (0.6–0.9)	8
CSF-IgM	mg/L	Median (IQR)		0.8 (0.3–1.2)	24	0.3 (0.1–0.6)	22	0.2 (0.1–0.3)	19	0.3 (0.2–0.4)	8
IgM index	CSF/S	Median (IQR)		0.1 (0.07–0.2)	24	0.09 (0.05–0.2)	22	0.06 (0.05–0.09)	19	0.07 (0.05–0.09)	8
IgM OCB	Yes/no		No	5/19	24	2/22	24	1/18	19	0/7	7
CSF-NfL	ng/L	Median (IQR)	<560	950 (470–3,500)	26	430 (320–560)	26	400 (300–560)	20	360 (320–440)	8

Abbreviation: HC = healthy control; IQR = interquartile range; OCB = oligoclonal bands.

Figure 1 Heat Map of 12 Dysregulated miRNAs in Multiple Sclerosis



At a false discovery rate, of $q = 0.05$, 12 miRNAs discriminated between pwMS before aHSCT (baseline, n = 33) and healthy controls (n = 29). In the heat map, red signifies higher normalized expression levels and blue signifies lower expression levels. Each miRNA is listed to the right of the heat map, corresponding to the row at which it is presented. Each participant is presented as a column. The patients were divided into groups based on their last prior treatment pre-aHSCT (first-line and second-line and naive). Black boxes in the heat map signify missing values. aHSCT = autologous hematopoietic stem cell transplantation; miRNA = microRNAs; pwMS = people with multiple sclerosis.

Correlation of CSF miRNA Levels With Clinical and Analytical Variables

Correlations between expression levels of the 12 dysregulated miRNAs and clinical variables were assessed with the Spearman rank correlation coefficient in samples obtained before aHSCT (Table 3).

Elevated expression levels of the 4 miRNAs that formed a cluster with strong associations with each other were associated with increasing numbers of gadolinium-enhancing lesions and relapses, as well as with increasing concentrations of mononuclear cells and NfL in the CSF.

Expression levels of miR-27b-3p were associated with NfL concentrations in CSF and the IgM index, wherein decreased expression was observed in pwMS with higher NfL concentrations and a higher IgM index. In a corresponding way, pwMS with a longer disease duration were observed to have lower expression levels of miR-143-3p.

CSF miRNAs in Patients With Gadolinium-Enhancing Lesions

PwMS with gadolinium-enhancing lesions at baseline (n = 13) exhibited significantly higher mean expression levels in 8 of the 12 miRNAs compared with pwMS without gadolinium-enhancing lesions (n = 20). The most important differences in mean expression levels between the groups were observed regarding miR-21-5p ($p < 0.0001$), miR-16-5p, miR-150-5p, and miR-146a-5p (p 0.001) (Figure 3).

CSF miRNA Expression and DMTs

To investigate whether miRNA expression levels before aHSCT were influenced by prior DMTs, the baseline group was divided into 3 subgroups based on their last prior treatment before aHSCT.

In an analysis of variance, 4 miRNAs (miR-16-5p, miR-21-5p, miR-150-5p, and miR-146a-5p) were identified to differ between the groups. In the post hoc analysis, expression levels of these 4 miRNAs were elevated in pwMS receiving first-line treatment (n = 11) compared with pwMS receiving second-line treatment (n = 18) ($p < 0.05$).

No differences were observed between either of the 2 treatment groups and the treatment-naïve group (n = 4). Overall, pwMS who received second-line treatment exhibited expression levels similar to HC, while pwMS who either received first-line treatment or were treatment-naïve exhibited altered expressions of the 12 miRNAs (Figure 1).

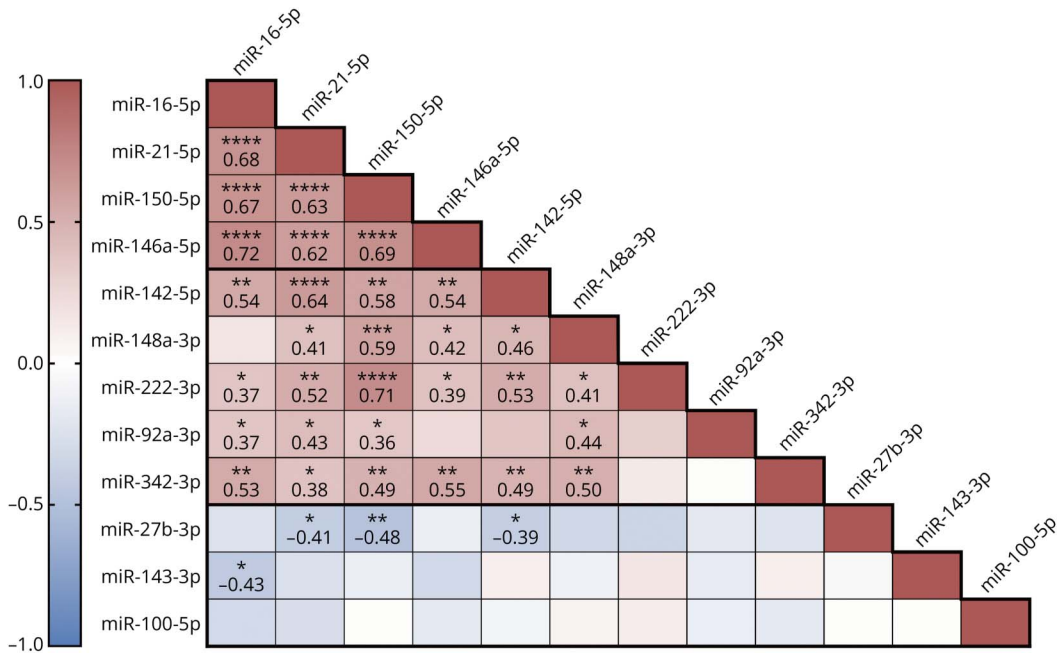
miRNA Alterations After Intervention With aHSCT for MS

After intervention with aHSCT, 4 of the 12 miRNAs were significantly altered at all follow-up timepoints compared with the individuals' own baseline sample, miR-16-5p, miR-21-5p, miR-150-5p, and miR-146a-5p (Figure 3, eFigure 1). Four other miRNAs were significantly altered at 1 or more, but not all, timepoints compared with the individuals' own baseline sample. miR-142-5p decreased at 1 year and 3–5 years after aHSCT (p <0.05), miR-222-3p at 1 year after aHSCT (p < 0.05), miR-92a-3p at 2 years after aHSCT ($p < 0.01$), and miR-148-3p ($p < 0.05$) at 3–5 years after aHSCT.

Dysregulated miRNAs and Biological Processes

We proceeded to analyze the biological relevance of miR-16-5p, miR-21-5p, miR-150-5p, and miR-146a-5p. Human molecules experimentally confirmed as targets for these 4 miRNAs of interest were obtained from miRTarBase (eTable 3). Subsequently, target lists were uploaded to Enrichr to investigate their involvement in gene ontology (GO) biological processes. Analysis of clustered GO terms unveiled the engagement of

Figure 2 Heat Map Depicting Correlation Matrix of the miRNAs



Pearson correlation was used to assess the covariation of the 12 significantly dysregulated miRNAs in multiple sclerosis. Bold text signifies correlation of statistical significance, and the asterisks signify the *p* values. miRNA = microRNAs.

miRNA-targeted genes in diverse biological processes, including regulation of apoptotic processes; IL-12, IL-17, and IL-23 production; inflammatory responses; myelin maintenance; and regulation of signaling cascades (Figure 4).

CSF miRNA Expression and Evidence of Inflammatory Disease Activity After aHSCT

The expression levels of the 12 dysregulated miRNAs were assessed in patients with EIDA (n = 8) and compared with patients who maintained NEDA-2 (n = 25) after intervention with aHSCT (eFigure 2). The levels of these miRNAs were similar at baseline and at first follow-up, 1 year after aHSCT. The expression of miR-142-5p and miR-342-3p was significantly elevated in patients with EIDA 2 years after aHSCT when compared with patients maintaining NEDA-2. Differences between the groups were also observed 3–5 years after aHSCT regarding expression levels of miR-100-5p, wherein elevated expression of miR-100-5p was observed among pwMS experiencing EIDA. The mean time following aHSCT at which EIDA was recorded was 3.5 years (SD 1.2).

Discussion

In this study, we identified a set of 12 miRNAs which were dysregulated in pwMS, particularly among patients with no treatment or first-line treatment. A recurring 4-miRNA signature, characterized by increased expression of miR-16-5p, miR-21-5p, miR-150-5p, and miR-146a-5p, consistently stood out in various analyses. Following intervention with aHSCT, the miRNA expression approached levels comparable with HCs, indicating a potent treatment effect.

The miRNAs comprising the 4-miRNA cluster were strongly associated with each other and with various clinical and analytical parameters and were normalized after intervention with aHSCT. The involvement of this 4-miRNA cluster in the pathophysiology of MS is further corroborated by previous reports. The most consistent findings have been reported with miR-150-5p dysregulation in CSF from pwMS.[5,16–18]

In all previous studies,[5,16–18] miR-150-5p expression was elevated in CSF samples from pwMS compared with control groups. One study reported decreased expression of miR-150-5p after introduction of natalizumab treatment and a simultaneous increase in the expression levels of miR-150-5p in plasma.[5] Another study found high expression levels of miR-150-5p in pwMS with lipid-specific oligoclonal IgM bands,[16] a biomarker suggested to indicate a more severe disease course. Arguably, their results along with the associations presented in this study between miR-150-5p and the number of relapses, number of gadolinium-enhancing lesions and CSF mononuclear cell counts, strengthen the link between miR-150-5p and inflammation. Moreover, the expression of this analyte seems to be particularly pronounced in patients with a more aggressive disease course.

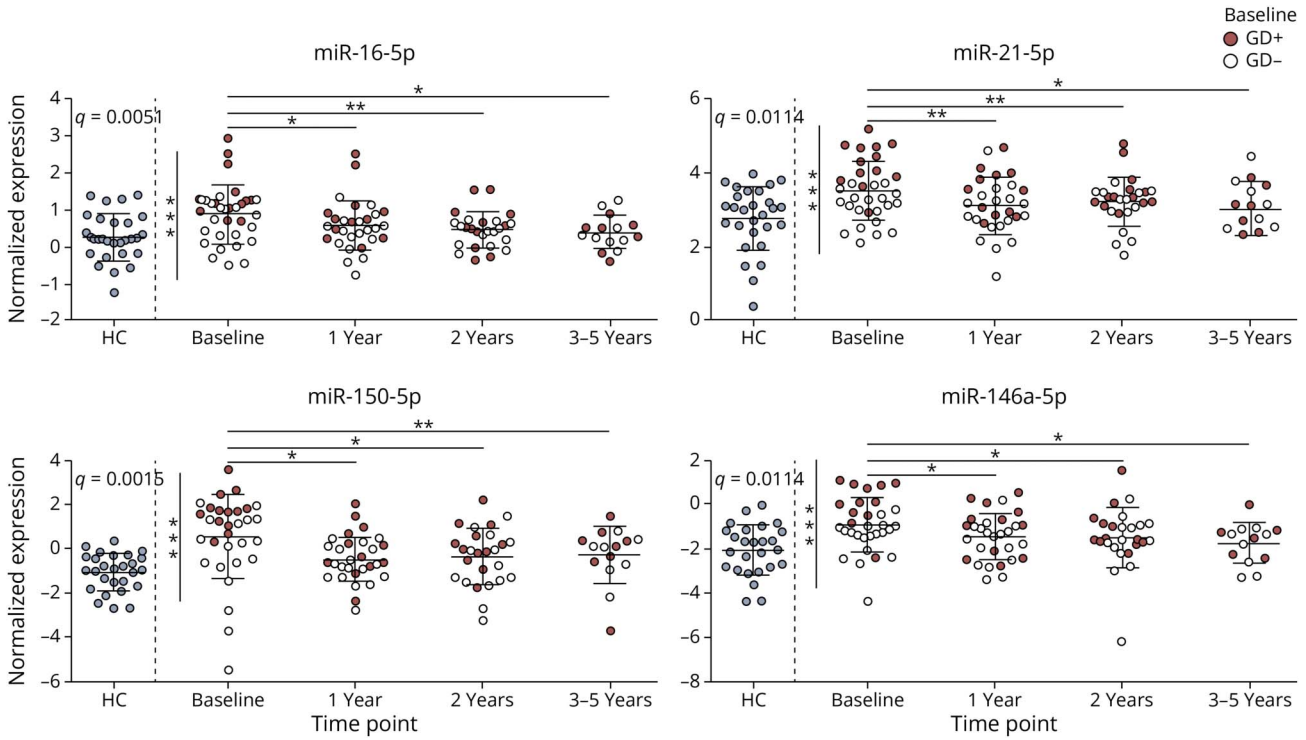
Most reports have described an upregulation of miR-146a-5p and miR-21-5p in CSF from pwMS,[4,17,19] with one notable exception.[16] It is difficult to reconcile these conflicting

Table 3 Spearman Rank Correlation Coefficient for Associations Between miRNAs and Clinical and Analytical Parameters

Clinical and analytical parameters																
	Spearman *r*	**Number of Gd + lesions at baseline**	**Mononuclear cell count**	**CSF-NfL**	**Number of relapses last year before aHSCT**	**P-IgG**	**Polynuclear cell count**	**IgM index**	**Disease duration**	**S-IgM**	**CSF-IgM**	**Age**	**CSF albumin**	**Albumin quota**	**CSF-IgG**	**IgG index**
miRNA	mir-16-5p	0.54[a]	0.52[a]	0.62[b]	0.38[c]	0.49[c]	−0.44[c]	0.28	−0.23	0.14	0.32	−0.19	0.11	−0.02	0.38	0.24
	mir-21-5p	0.72 [d]	0.57[a]	0.42[c]	0.60[b]	0.23	−0.40[c]	0.35	−0.09	0.23	0.41[c]	−0.15	0.04	−0.06	0.14	0.15
	mir-150-5p	0.59[b]	0.64[b]	0.59[a]	0.42[c]	0.40[c]	−0.34	0.30	−0.12	0.18	0.31	−0.08	0.12	0.06	0.26	0.22
	mir-146a-5p	0.71 [d]	0.64[b]	0.45[c]	0.59[b]	0.20	−0.36	0.05	−0.14	0.17	0.07	0.12	0.16	0.00	0.31	0.29
	mir-142-5p	0.56[a]	0.55[c]	0.48[c]	0.35	0.51[c]	−0.30	0.08	−0.32	0.33	0.29	−0.22	0.09	0.05	0.34	0.19
	mir-222-3p	0.45[c]	0.65[b]	0.27	0.29	0.29	0.08	0.31	0.01	0.00	0.36	−0.02	0.16	0.17	0.25	0.01
	mir-148a-3p	0.48[a]	0.06	0.40	0.33	0.09	−0.17	0.13	−0.11	0.29	0.16	−0.06	0.08	0.07	0.17	0.21
	mir-92a-3p	0.55[b]	0.26	0.33	0.33	0.28	−0.06	0.43[c]	0.01	0.13	0.31	−0.09	−0.14	−0.21	−0.05	0.01
	mir-342-3p	0.36[c]	0.43[c]	0.14	0.21	0.19	0.01	−0.15	−0.13	0.06	−0.08	−0.09	0.01	0.07	0.16	−0.06
	mir-27b-3p	−0.12	−0.34	−0.47[c]	−0.11	−0.29	0.38	−0.52[a]	0.27	0.11	−0.27	0.32	0.04	0.09	−0.03	−0.11
	mir-143-3p	−0.22	−0.15	0.06	−0.18	0.05	0.14	−0.01	−0.43[c]	−0.05	−0.21	−0.15	−0.22	−0.14	−0.29	−0.12
	mir-100-5p	−0.15	0.02	−0.12	0.10	0.15	0.21	−0.13	0.07	0.40[c]	0.20	0.02	−0.14	−0.15	−0.03	−0.04

Abbreviation: aHSCT = autologous hematopoietic stem cell transplantation.
[a] *p* values < 0.01.
[b] *p* values < 0.001.
[c] *p* values < 0.05.
[d] *p* values < 0.0001.

Figure 3 Four miRNAs Significantly Decrease at all Timepoints After aHSCT

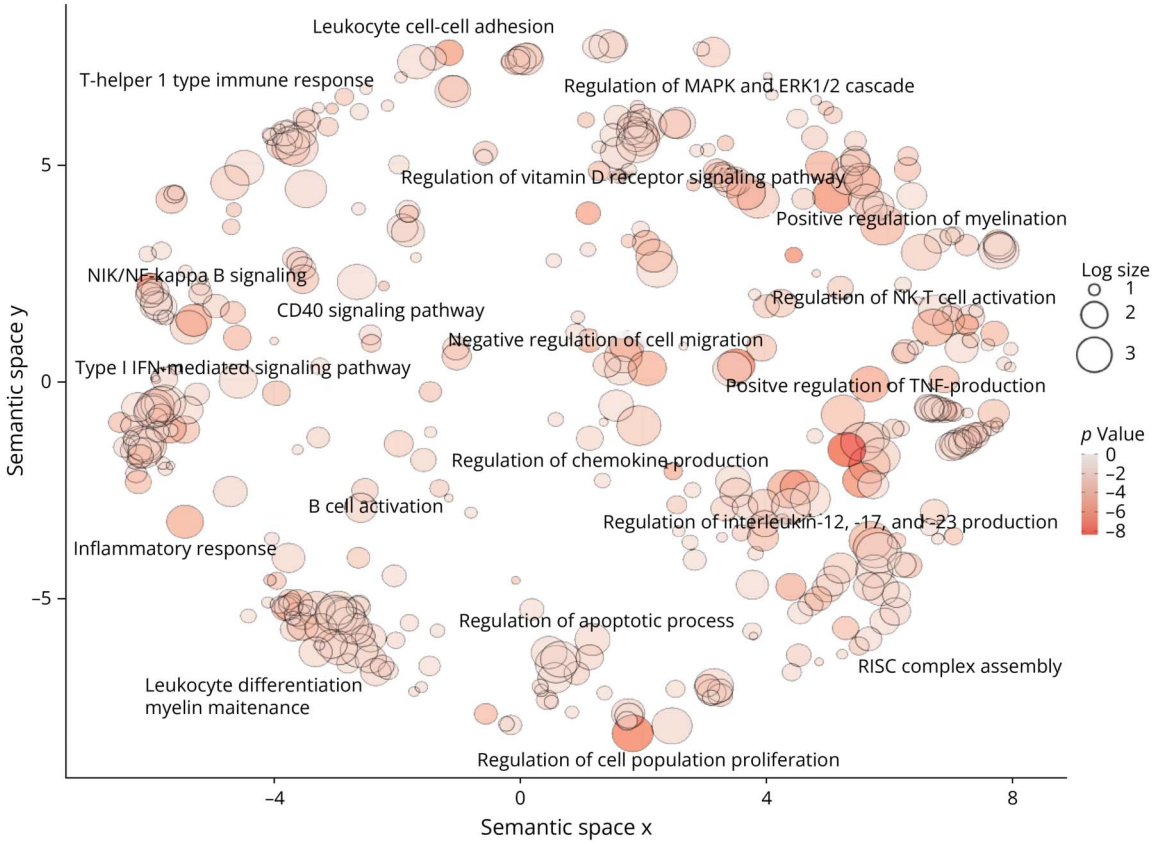


Four of the 12 miRNAs, discriminating pwMS from healthy controls (HC), recurred in multiple analyses. Depicted above are the expression levels of the miRNAs before (baseline) and at yearly follow-ups after (1, 2, and 3–5 years) aHSCT. Red dots signify pwMS with gadolinium-enhancing lesions (Gd+) at baseline. Error bars signify the mean and SD. Horizontal asterisks signify paired analyses comparing pwMS after aHSCT with their own sample at baseline. Vertical asterisks signify differences between pwMS with (Gd+) and without (Gd-) gadolinium-enhancing lesions at baseline. aHSCT = autologous hematopoietic stem cell transplantation.

findings, but they could possibly be attributed to differences in cohorts, control groups, method differences, and/or sample handling before analysis.[16] In support of upregulation of miR-146a-5p and miR-21-5p in pwMS, elevated expression levels of both miR-146a-5p and miR-21-5p were also observed in active white matter lesions in brain tissue sections. Furthermore, an association with the number of gadolinium-enhancing lesions has been described,[19,20] corroborating our results which showed strong correlations with the number of gadolinium-enhancing lesions. Until now, miR-16-5p has not been highlighted as a CSF miRNA of interest in MS, although one study described increased expression of miR-16-5p in peripheral blood mononuclear cell from pwMS before intervention with aHSCT, with a subsequent normalization after treatment.[21] Notably, a bioinformatic study found miR-16-5p to be the most common miRNA in regulating common hub genes in both MS and Alzheimer disease (AD).[22]

Beyond the 4-miRNA cluster, miR-143-3p and miR-27b-3p were also found to associate with clinical and analytical factors. PwMS with decreased expression levels of these miRNAs were observed to have had a longer disease duration and higher NfL concentrations in the CSF. Previous CSF studies have reported that the expression levels of miR-143-3p, which was the only miRNA to correlate with disease duration, were significantly lower in people with progressive disease.[3,4] Functionally, miR-143-3p has been discussed to have a neuroprotective role and an effect on neuronal repair.[23] In addition, higher miR-143-3p expression has been observed in astrocytes compared with microglia, neurons, and oligodendrocytes.[24] Decreased expression levels of miR-143-3p in the CSF from pwMS could potentially be a biomarker of neurodegeneration and the phenotypic shift from RR-MS to secondary progressive MS, but this would need to be studied in greater detail to elucidate which factors potentiate the decrease in miR-143-3p expression and then confirmed in larger cohorts. Decreased expression of miR-27b-3p was observed in pwMS with a higher IgM-index and higher NfL concentrations in the CSF. Elevated expression levels of miR-27b-3p have been associated with alleviation of inflammation through regulation of macrophage polarization through degrading macrophage colony-stimulating factor-1 (CSF-1), potentially interfering with exacerbation of inflammation in autoimmune diseases.[25] A target analysis of miR-27b-3p and miR-143-3p revealed a shared target in CD44, which is implicated in many biological processes such as response to neuronal injury and is reported to be a shared hub gene for both AD and MS.[22,26] It has been reported that CD44 is overexpressed in CSF-derived monocytes, especially in pwMS who exhibit gadolinium-enhancing lesions.[27] One possibility is that increased

Figure 4 Enrichment Analysis of the 4 Distinct miRNAs



Functional gene enrichment with miRTarBase revealed affected genes by the 4 distinct miRNAs. GO searches in EnrichR revealed biological processes affected by the miRNAs of interest. Biological processes of interest were visualized with REVIGO and are displayed in the graph above with *p* values of the biological process as well as the logarithmic size of the semantic term. GO = gene ontology; miRNA = microRNAs.

expression of CD44 occurs as a response to neuroaxonal damage and the resulting decreased expression of miR-27b-3p and miR-143-3p is an epiphenomenon of this response.

Twelve miRNAs were observed to be dysregulated in CSF from pwMS. Of these 12 miRNAs, 10 were upregulated and 2 downregulated compared with HCs. After intervention with aHSCT, we observed changes in expression levels in 8 of the 12 miRNAs all of which were among the upregulated miRNAs at baseline. Furthermore, the 4-miRNA signature with overexpression of miR-16-5p, miR-21-5p, miR-150-5p, and miR-146a-5p before aHSCT was the only miRNAs that decreased after aHSCT and remained low throughout the follow-up period. Our results further strengthen the potent treatment effect of intervention with aHSCT[8,28] but also shed light on the potential of biomarkers in representing inflammatory disease activity, progression, and monitoring relapses after aHSCT.

We performed an enrichment analysis of the previously identified 4-miRNA signature, which revealed several processes regulated by this miRNA cluster, including vitamin D signaling and myelin maintenance. Although the links between myelination regulation, vitamin D signaling, and MS pathophysiology are well established, the association between these processes and the 4-miRNA cluster underscores the broader effect these molecules may have on MS pathophysiology. Notably, the cluster was also implicated in the regulation of T helper (Th) 1 type immune responses, leukocyte cell-cell adhesion, and NF-κB signaling, all previously implicated in propagation of autoimmune processes in MS. In addition, we found that the 4-miRNA cluster is involved in the regulation of interleukin-12, IL-17, and IL-23. In a previous study[29] on the same cohort, our group observed elevated concentrations of IL-12B, a subunit of IL-12, in CSF from people with RRMS compared with both HCs and people with primary progressive MS. This strengthens the association between the 4-miRNA cluster and IL-12B, described in this study.

Previous research has shown reductions in peripheral blood Th17 cells and IL-17A production after aHSCT for MS, with no changes in IL-23 levels,[30] while another study reported decreases in both IL-17A and IL-23 at all long-term follow-up points after aHSCT.[31] Altogether, this suggests an interactive association between dysregulated cytokines IL-12, IL-17, and

IL-23; pathogenic Th17 cells; and the expression levels of miR-16-5p, miR-21-5p, miR-150-5p, and miR-146a-5p in MS affecting central processes to disease pathophysiology. An association evidently efficiently targeted by intervention with aHSCT.

Although the precise mechanism by which aHSCT induces altered miRNA expression remains unclear, a previous study proposed that these changes might result from an enhanced immunoregulatory network following the intervention.[21] However, the observed correlations between the 4-miRNA cluster and factors such as mononuclear cell counts and gadolinium-enhancing lesions suggest that altered miRNA expression could also arise from a reduction in infiltrating pathogenic immune cells or decreased cellular stress within the CNS. Further research is necessary to determine the origin of these miRNAs and to clarify how aHSCT exerts these profound effects on MS pathophysiology.

Of the 12 miRNAs, detected to be dysregulated at baseline, merely 2 miRNAs stood out in samples from pwMS experiencing EIDA after aHSCT compared with pwMS maintaining NEDA-2. The expression levels of both miR-142-5p and miR-342-3p were increased at 2 years of post-aHSCT in pwMS with EIDA. Both have previously been described to be dysregulated in pwMS,[4,32] but never in connection to recurring disease after aHSCT. Previous studies have reported the increased expression of miR-142 to be associated with inflammation, possibly through suppression of regulatory T-cell activity.[21,33] Biological processes reported to be associated with miR-342-3p include neuroaxonal injury.[32] Considering that the elevated expression of the 2 miRNAs was detected in samples collected at the 2-year follow-up, thereby preceding the mean time at which EIDA was clinically recorded by 1.5 years, this could indicate that they are early markers of disease activity and relapse. However, this would need to be confirmed in a larger cohort.

In the past decade, dysregulation of miRNAs in pwMS has been reported in a number of studies, both in serum and CSF, with more and more candidates being discovered and discussed. However, the candidates of interest often differ between studies, and the findings of previous studies are rarely validated, which in itself is a limitation of the field of biomarker research on miRNAs.

This study also had some limitations. The main methodological limitation of this study is that the discovery cohort comprised merely 8 participants with 4 in each group. This could introduce bias in the selection of the final 22 miRNA candidates for the validation, potentially missing valuable candidates that were not expressed in the selected representatives. However, the restriction in the size of the discovery cohort was a matter of cost. In addition, of the initial 187 samples, 30 samples did not pass quality control. This subsequently resulted in additional sample exclusion; in case a baseline sample did not pass quality control, all related samples were excluded. This strict exclusion approach could possibly have concealed interesting and beneficial findings. Other methodological limitations to consider are the choice of sample matrix. Although CSF is preferable due to the proximity to the brain; serum and plasma are more accessible and less invasive for the participant, thereby also incentivizing research to focus on plasma/serum biomarkers, culminating in CSF results being less generalizable. Finally, although we performed an enrichment analysis of involvement in gene regulation of the cluster of 4 miRNAs, the analysis does not elucidate the direction of the regulation, whether it be positive or negative regulation.

miRNAs are a novel class of promising biomarkers for clinical applications owing to their stability and detectability across various tissues and biological fluids. We identified a CSF signature of 4 miRNAs which were increased in pwMS and associated with many clinical and analytical factors. This 4-miRNA signature is involved in regulation of the inflammatory response and myelin maintenance. The levels of all these miRNAs decreased following intervention with aHSCT, approaching the levels of HCs. The miRNA levels then remained stable after aHSCT throughout the follow-up period. Our study adds to the growing body of literature on CSF miRNA in MS and further strengthens the evidence base for the therapeutic efficacy of aHSCT for MS.

Author Contributions

I. Pavlovic: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data. F. Axling: drafting/revision of the manuscript for content, including medical writing for content; analysis or interpretation of data. F.H. Nazir: drafting/revision of the manuscript for content, including medical writing for content. M. Müller: drafting/revision of the manuscript for content, including medical writing for content. A. Wiberg: drafting/revision of the manuscript for content, including medical writing for content; study concept or design. J. Burman: drafting/revision of the manuscript for content, including medical writing for content; major role in the acquisition of data; study concept or design; analysis or interpretation of data.

Study Funding

This work was supported by the Swedish Research Council (2021–02814), Neuro Sweden, Bissen Brainwalk Foundation, Marcus and Marianne Wallenberg Foundation, the Swedish Society for Medical Research, and the Swedish Society for Medicine (SLS-726341). The funding agencies did not influence the design and conduct of the study; collection, management, analysis, and interpretation of the data; preparation, review, or approval of the manuscript; and decision to submit the manuscript for publication.

Disclosure

The authors report no relevant disclosures. Go to Neurology.org/NN for full disclosures.

Publication History

Received by *Neurology: Neuroimmunology & Neuroinflammation* August 23, 2024. Accepted in final form October 22, 2024. Submitted and externally peer reviewed. The handling editor was Deputy Editor Scott Zamvil, MD, PhD, FAAN.

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