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Identification of MS-specific serum miRNAs in an international multicenter study

Keren Regev, MD, Brian C. Healy, PhD, Anu Paul, PhD, Camilo Diaz-Cruz, MD, Maria Antonietta Mazzola, MD, Radhika Raheja, PhD, Bonnie I. Glanz, PhD, Pia Kivisäkk, MD, PhD, Tanuja Chitnis, MD, Maja Jagodic, PhD, Fredrik Piehl, MD, PhD, Tomas Olsson, MD, Mohsen Khademi, PhD, Stephen Hauser, MD, Jorge Oksenberg, PhD, Samia J. Khoury, MD, Howard L. Weiner, MD, and Roopali Gandhi, PhD

Correspondence
Dr. Gandhi
rgandhibwh@gmail.com

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Abstract

Objective
To identify circulating microRNAs (miRNAs) linked to disease, disease stage, and disability in MS across cohorts.

Methods
Samples were obtained from the Comprehensive Longitudinal Investigation of Multiple Sclerosis (CLIMB, Boston, MA), EPIC (San Francisco, CA), AMIR (Beirut, Lebanon) as part of the SUMMIT consortium, and Stockholm Prospective Assessment of Multiple Sclerosis (Stockholm, Sweden) cohorts. Serum miRNA expression was measured using locked nucleic acid–based quantitative PCR. Four groups were compared: (1) MS vs healthy control (HC), (2) relapsing-remitting (RR) vs HC, (3) secondary progressive (SP) vs HC, and (4) RR vs SP. A Wilcoxon rank-sum test was used for the comparisons. The association between each miRNA and the Expanded Disability Status Scale (EDSS) score was assessed using the Spearman correlation coefficient. For each comparison, the p values were corrected for multiple comparisons using the approach of Benjamini and Hochberg to control the false discovery rate.

Results
In the CLIMB cohort, 5 miRNAs (hsa-miR-484, hsa-miR-140-5p, hsa-miR-320a, hsa-miR-486-5p, and hsa-miR-320c) showed a significant difference between patients with MS and healthy individuals; among these, miR-484 remained significant after accounting for multiple comparisons ($p = 0.01$). When comparing RRMS with HCs, hsa-miR-484 showed a significant difference ($p = 0.004$) between the groups after accounting for multiple group comparisons. When SP and HC were compared, 6 miRNAs (hsa-miR-484, hsa-miR-140-5p, hsa-miR-142-5p, hsa-miR-320a, hsa-miR-320b, and hsa-miR-320c) remained significantly different after accounting for multiple comparisons. Disability correlation analysis with miRNA provided 4 miRNAs (hsa-miR-320a, hsa-miR-337-3p, hsa-miR-199a-5p, and hsa-miR-142-5p) that correlated with the EDSS during the internal reproducibility phase. Among these, hsa-miR-337-3p was the most statistically significant miRNA that negatively correlated with the EDSS in three of the MS cohorts tested.

Conclusions
These findings further confirm the use of circulating serum miRNAs as biomarkers to diagnose and monitor disease status in MS.

Classification of evidence
This study provides Class III evidence that levels of circulating miRNAs identify patients with MS.

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From the Partners Multiple Sclerosis Center (K.R., B.C.H., A.P., M.A.M., R.R., P.K., T.C., H.L.W., R.G.), Brigham and Women's Hospital; Department of Neurology, Harvard Medical School (B.C.H., C.D.-C., B.I.G., T.C., H.L.W, R.G.), Ann Romney Center for Neurologic Diseases, Brigham and Women's Hospital; Biostatistics Center (B.C.H.); Massachusetts General Hospital, Boston, MA; Department of Clinical Neuroscience, Neuroimmunology Unit (M.J., F.P., T.O., M.K.), Karolinska Institute, Stockholm, Sweden; Department of Neurology (S.H., J.O.), School of Medicine, University of California, San Francisco; and Nehme and Therese Tohme Multiple Sclerosis Center (S.J.K.), Faculty of Medicine, American University of Beirut Medical Center, Lebanon.

Authors in the SUMMIT Consortium are listed at http://links.lww.com/NXI/A75.

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Glossary

AD = Alzheimer disease; **AMIR** = AUBMC Multiple Sclerosis Interdisciplinary Research; **ALS** = amyotrophic lateral sclerosis; **AUBMC** = American University of Beirut Medical Center; **AUC** = Area under curve; **AUCROC** = area under the ROC curve; **CLIMB** = Comprehensive Longitudinal Investigation of Multiple Sclerosis; **EPIC study** = Expression, Proteomics, Imaging, Clinical study; **FDR** = false discovery rate; **HC** = healthy control; **miRNA** = microRNA; **RA** = rheumatoid arthritis; **RR** = relapsing-remitting; **RRMS** = relapsing-remitting multiple sclerosis; **SPMS** = secondary progressive multiple sclerosis; **STOPMS II** = Stockholm Prospective Assessment of Multiple Sclerosis.

MS is an autoimmune disorder that attacks the CNS.[1,2] The disease course of MS is extremely variable[3,4]; this diversity in the disease phenotype is not well correlated with currently used biomarkers; thus, there is an urgent need for efficacious diagnostic assays for MS detection, prognostic measures of disease progression, and treatment response.

Research on circulating biomarkers has yielded some promising candidates for MS detection including glycoproteins, chemokines, antibodies, lipopeptides, and genetic polymorphisms. More recently, microRNAs (miRNAs) have been investigated as potential biomarkers in MS.[5–8] miRNAs constitute particularly exciting biomarker candidates because they are very stable molecules, and their differential expression in circulating fluids (e.g., blood) has been shown to correlate with many disease states, including MS.[5]

Recently, our laboratory identified a set of serum miRNAs as potential diagnostic MS biomarkers.[9] Differential expression of 7 miRNAs in MS vs healthy controls (HCs) was validated during a 2-year comprehensive discovery and validation study. In addition, we reported 10 miRNAs that significantly correlated with patient disability status as measured by the Expanded Disability Status Scale (EDSS).[9]

In the current study, we tested the reproducibility of our previous findings using patients with MS and HCs from the Comprehensive Longitudinal Investigation of Multiple Sclerosis (CLIMB) cohort. In addition, we tested the transportability of our results in MS samples collected from three other international MS collections including the SUMMIT consortium (San Franciso and Beirut) and the STOPMS II study (Stockholm).

This study design using patient samples from multiple centers (n = 259) was implemented to identify the most promising serum miRNA biomarker candidates for MS diagnosis and disability status.

Methods

Study design

The study involved multiple phases: (1) discovery, (2) validation, (3) reproducibility, and (4) transportability phases (figure e-1, links.lww.com/NXI/A63). The first 2 phases of the study have been described previously.[9] These phases are summarized as follows:

1. Discovery: 652 miRNAs were measured in participants from 3 groups: RRMS (n = 7), SPMS (n = 9), and HC (n = 20).[9]
2. Validation: based on the findings observed in the discovery phase, 191 miRNAs were measured in a larger validation set of participants from CLIMB patients: RRMS (n = 29), SPMS (n = 19), and HC (n = 30), including an additional cohort of participants with either other inflammatory diseases or other neurodegenerative diseases.[9] This phase was used as the training set for a multivariate approach using predictive models.
3. Reproducibility phase: 73 miRNAs showing promise to serve as biomarkers[9] were further analyzed in a reproducibility phase with RRMS (n = 24), SPMS (n = 18), and HC (n = 30) from the CLIMB cohort. This phase was used as the test set for the predictive models that were designed in the validation (training phase).
4. Transportability phase: the 73 miRNAs from the reproducibility phase were further analyzed in 3 external cohorts with study participants from 3 MS centers worldwide: RRMS (n = 91), SPMS (n = 33), and HC (n = 58).

Patient selection

For the reproducibility phase, a new group of participants from the CLIMB cohort was identified. In addition to this new group of CLIMB participants, 3 external cohorts were included in the study. The first cohort was from the AUBMC-Multiple Sclerosis Interdisciplinary Research cohort of the American University of Beirut Medical Center—Abou Haidar Neuroscience Institute; 31 patients with RRMS, 5 patients with SPMS, and 19 HCs were included in the study. The second cohort was from the EPIC (Expression/genomics, Proteomics, Imaging, Clinical) cohort of the University of California at San Francisco[10]; 27 patients with RRMS, 7 patients with SPMS, and 26 HCs were included in the study. The third cohort was from the STOPMS II (Stockholm Prospective Assessment of Multiple Sclerosis) cohort of Sweden; 33 patients with RRMS, 21 patients with SPMS, and 13 HCs were included in the study.

The demographic characteristics of all 4 cohorts are provided in table 1. None of the selected patients were on any disease-modifying treatment at the time of sample collection.

Table 1 Patient demographics

	Relapsing-remitting MS	Secondary progressive MS	Healthy controls
CLIMB			
N	24	18	30
Age	32.4 (6.6)	56.4 (9.3)	45.4 (15)
Female	19 (0.792)	15 (0.833)	25 (0.833)
EDSS	0.6 (0.9)	5.5 (1.6)	
DD in y	3.9 (2.4)	17.2 (5.9)	
AMIR			
N	31	5	19
Age	33.5 (10.2)	40.2 (7.8)	37.6 (11.2)
Female	21 (0.677)	2 (0.4)	12 (0.632)
EDSS	2.1 (1.2)	4.5 (1.4)	
DD	4.6 (4.7)	15.2 (11)	
EPIC			
N	27	7	26
Age	37.6 (6.8)	52.7 (5.8)	45.5 (10.2)
Female	20 (0.741)	7 (1)	12 (0.462)
EDSS	1 (0.8)	5.1 (1.3)	
DD	3.9 (2.4)	19 (6)	—
STOPMS II			
N	33	21	13
Age	33.1 (9.4)	43.2 (4.3)	30.9 (5.6)
Female	23 (0.697)	11 (0.524)	6 (0.462)
EDSS	1.66 (1.7)	5.3 (1.5)	
DD	2.2 (3.4)	19.6 (5.2)	—

Abbreviations: AMIR = AUBMC-Multiple Sclerosis Interdisciplinary Research cohort; CLIMB = Comprehensive Longitudinal Investigation of Multiple Sclerosis; DD = disease duration; EDSS = Expanded Disability Status Scale; EPIC = Expression/genomics, Proteomics, Imaging, and Clinical cohort; STOPMS II = Stockholm Prospective Assessment of Multiple Sclerosis.

Standard Protocol Approvals, Registrations, and Patient Consents

Written informed consent was obtained from all patients, and regional ethical committees approved the study (CLIMB cohort, IRB Protocol No: 1999P010435; EPIC cohort, IRB Protocol No: 10-00104; AMIR cohort, IRB Protocol No: IM.SK1.01; and STOPMS II cohort, IRB Protocol No: 2010/2:1).

Sample collection and storage

Blood samples were collected in glass red top serum vacutainer tubes without additives (Becton, Dickinson and Company, Franklin Lakes, NJ), and serum tubes were kept at room temperature for 30–60 minutes. Each sample was centrifuged at 2000 RPM for 10 minutes to separate the serum and then stored at −70°C until RNA extraction. Freezing of the serum occurred within 2 hours of the blood draw. Serum samples from the external cohorts were sent to Boston for further processing.

RNA extraction and analysis

RNA was isolated using the miRCURY kit (Exiqon, Waltham, MA) and converted to cDNA using a synthesis kit from Exiqon following the manufacturer's instructions. Prepared cDNAs were stored at −20°C until use. LNA SYBR green–based real-time PCR was used to detect the expression of selected miRNAs (Exiqon). Normalization was performed using the mean expression of the miRNAs with the best stability index, i.e., showing expression stability in our sample set. NormFinder software was used to calculate the stability index. We used 5 normalizing miRNAs (hsa-miR-484, hsa-miR-30e-5p, hsa-miR-590-5p, hsa-let-7d-3p, and hsa-miR-15b-5p). The formula used to calculate the normalized Cq values is as follows: Normalized Cq = average Cq − assay Cq.

Statistical analysis

In the CLIMB cohort, 4 groups were compared for each miRNA: (1) all MS vs HC, (2) relapsing-remitting (RR) vs HC, (3) secondary progressive (SP) vs HC, and (4) RR vs SP, and a Wilcoxon rank-sum test was used for the comparisons. For the Wilcoxon rank-sum test, participants that had no measured expression were assigned a value below the smallest available measurement so that these participants had the smallest rank for all analyses. In addition to the group comparisons, the association between each miRNA and the EDSS score was assessed using the Spearman correlation coefficient. For each comparison, the p values for miRNAs that passed validation were corrected for multiple comparisons using the approach of Benjamini and Hochberg[11] to control the false discovery rate (FDR).

We also compared the miRNA expression level between the groups using a proportional odds model to adjust for age and sex. The proportional odds model is a generalization of the Wilcoxon rank-sum test that allows adjustment for other variables.

In addition to the individual miRNA comparisons, prediction models were developed in the validation cohort (training set). The prediction models were built using all miRNAs that were in the same direction in the discovery and validation phases of the study using best subset regression with 3 miRNAs in the model. Three miRNAs were chosen, given the sample size limitations in the validation set (training set). The models were evaluated using the area under the ROC curve (AUCROC) in both the validation cohort (training set) and the reproducibility cohort (test set) to estimate the ability of the model to discriminate between the 2 groups. The estimated AUCROC from the reproducibility phase of the CLIMB cohort provides a more reliable estimate because data

used in this set were not used in model building. In addition to best subset regression, several other approaches were used to build prediction models including stepwise selection, lasso, decision trees, and random forests, and the predictive performance of these approaches in our test set was assessed.

All analyses performed in the CLIMB cohort were completed in the 3 additional MS centers' cohorts during transportability phase. The miRNAs that showed significant differential expression or a significant association with the EDSS in multiple cohorts were identified. Furthermore, the AUCROC for each of the predictive models built in the CLIMB validation cohort (training set) was calculated for each external test set.

Data availability

Anonymized data not provided in the article because of space limitation will be shared upon request of other qualified investigators.

Results

Reproducibility of biomarkers in the CLIMB cohort

The miRNAs that qualified the validation of the comparison between MS and HC in the CLIMB cohort with $p < 0.05$ were tested during the reproducibility phase (table 2). Five previously validated miRNAs, namely, hsa-miR-484, hsa-miR-140-5p, hsa-miR-320a, hsa-miR-486-5p, and hsa-miR-320c, showed a significant difference, and 1 miRNA remained significant after accounting for multiple comparisons (hsa-miR-484, $p = 0.01$). When multiple miRNAs were included in a predictive model for MS vs HC, the miRNAs included were hsa-let-7c-5p, hsa-miR-452-5p, and has-miR-484. The AUCROC for this model in the validation cohort was 0.85, whereas the AUCROC in the reproducibility phase was 0.70 (figure e-2, links.lww.com/NXI/A63). When the other model building approaches were used, the AUCROC in the reproducibility cohort ranged from 0.67 to 0.71.

The comparisons of the miRNAs between RR and HC are provided in (table e-1, links.lww.com/NXI/A64). In this comparison, only hsa-miR-484 showed a significant difference ($p = 0.004$) between the groups after accounting for multiple comparisons. When best subset regression was used to select a model for this comparison, the miRNAs included in the model were hsa-miR-15b-3p, hsa-miR-451a, and hsa-miR-584-5p. The AUCROC for this model in the validation set was 0.82, whereas the AUCROC in the reproducibility phase was 0.68 (figure e-3, links.lww.com/NXI/A63). When the other model building approaches were used, the AUCROC in the validation ranged from 0.63 (decision tree) to 0.68 (best subset regression).

When SP and HC were compared (table e-2, links.lww.com/NXI/A64), 8 miRNAs were significantly different, and 6 miRNAs (hsa-miR-484, hsa-miR-140-5p, hsa-miR-142-5p, hsa-miR-320a, hsa-miR-320b, and hsa-miR-320c) were significantly different after accounting for multiple comparisons. The miRNAs included in the model selected by best subset regression were hsa-let-7c-5p, hsa-miR-320a, and hsa-miR-424-5p. The AUCROC for this model in the validation was 0.99, whereas the AUCROC in the reproducibility phase was 0.71 (figure e-4, links.lww.com/NXI/A63). When the other model building approaches were used, the AUCROC in the validation ranged from 0.71 (best subsets) to 0.75 (random forests).

When RR and SP were compared (table e-3, links.lww.com/NXI/A64), no previously validated miRNAs were significantly different between the groups. When best subset regression was used to build a model, the miRNAs included in the model were hsa-miR134-5p, hsa-miR-337-3p, and hsa-miR-532-3p. The AUCROC for this model in the validation was 0.93, whereas the AUCROC in the reproducibility phase was 0.63 (figure e-5, links.lww.com/NXI/A63). When the other model building approaches were used, the AUCROC in the validation ranged from 0.53 (decision tree) to 0.72 (lasso).

Table 2 Differential expression of significant miRNAs during the reproducibility phase (MS vs healthy controls)

	Reproducibility phase								Regev et al., 2016
	Number Expressed		Mean Expression		Fold Change				
miRNA	MS	HC	MS	HC	MS:HC	Wilcoxon *p* value	FDR value	Adjusted *p* value[a]	Wilcoxon *p* value
hsa-miR-484	42	30	−0.38	−0.66	1.21	0.0004	0.01	<0.01	<0.01
hsa-miR140-5p	42	30	−2.76	−2.41	0.78	0.01	0.07	0.01	<0.01
hsa-miR-320a	42	30	1.62	1.3	1.25	0.02	0.08	0.02	<0.01
hsa-miR486-5p	42	30	2.67	2.04	1.55	0.02	0.08	0.01	<0.01
hsa-miR-320c	42	30	−2.51	−2.89	1.30	0.04	0.12	0.04	<0.01

Abbreviations: FDR = false discovery rate; HC = healthy control; miRNA = microRNA.
Fifteen miRNAs were tested, and hsa-miR-320b, hsa-miR-20a-5p, hsa-miR-25-3p, hsa-miR-365a-3p, hsa-miR-941, hsa-miR-584-5p, hsa-miR-143-3p, hsa-miR-140-3p, hsa-let-7c-5p, and hsa-let-7g-5p were not significant during the reproducibility phase.
Fold change was calculated as 2(differences in mean).
[a] Adjusted *p* value from proportional odds model adjusting for age and sex.

Table 3 Correlation of significant miRNAs in association with Expanded Disability Status Scale in the reproducibility phase

	Reproducibility Phase			Regev et al., 2016	
miRNA	**Spearman estimate**	***p* Value**	**FDR**	**Spearman estimate**	***p* Value**
hsa-miR-142-5p	−0.31	0.05	0.17	−0.39	0.00
hsa-miR-199a-5p	−0.34	0.03	0.17	−0.32	0.00
hsa-miR-320b	0.41	0.01	0.06		
hsa-miR-337-3p	−0.41	0.01	0.06	−0.34	0.00

Abbreviations: FDR = false discovery rate; miRNA = microRNA.
Fifteen miRNAs were tested, and hsa-miR-140-3p, hsa-miR-142-3p, hsa-miR-16-5p, hsa-miR-199a-3p, hsa-miR25-3p, hsa-miR27a-3p, hsa-miR301b, hsa-miR-320a, hsa-miR-376b-3p, hsa-miR-486-5p, and hsa-miR-877-5p were not significant during the reproducibility phase.

For the disability biomarkers, the miRNAs that were significantly correlated with the EDSS in validation as published in Regev et al. 2016[9] were further measured in reproducibility phase cohort. We found that 4 miRNA, hsa-miR-320b, hsa-miR-337-3p, hsa-miR-199a-5p, and hsa-miR-142-5p (table 3), were significantly correlated with the EDSS in the reproducibility phase cohort.

Transportability of biomarkers in external MS cohorts

When the additional cohorts were analyzed, a modest number of miRNAs showed significant differential expression in the same direction in multiple cohorts (table 4). For the comparison of MS and HC, 5 miRNAs were found to be

Table 4 miRNAs showing significant association/differential expression during the transportability phase

miRNAs	
	Sites with significant differential expression in MS vs HC
hsa-let-7e-5p	AMIR ($p = 0.032$), STOPMS II ($p < 0.01$)
hsa-let-7f-5p	CLIMB ($p = 0.05$), STOPMS II ($p = 0.002$)
hsa-miR-486-5p	CLIMB ($p = 0.02$), STOPMS II ($p = 0.013$)
hsa-miR-30e-5p	CLIMB ($p = 0.013$), EPIC ($p = 0.047$)
	Sites with significant differential expression in RR vs. HC
hsa-let-7e-5p	AMIR ($p = 0.031$), STOPMS II ($p = 0.002$)
hsa-let-7f-5p	CLIMB ($p = 0.038$), STOPMS II ($p = 0.001$)
	Sites with significant differential expression in SP vs. HC
hsa-miR-320a	CLIMB ($p = 0.005$), STOPMS II ($p = 0.018$)
hsa-miR-320b	CLIMB ($p = 0.002$), STOPMS II ($p = 0.01$)
hsa-miR-320c	CLIMB ($p = 0.009$), STOPMS II ($p = 0.046$)
hsa-miR-486-5p	CLIMB ($p = 0.023$), STOPMS II ($p = 0.016$)
	Sites with significant correlation between miRNA and EDSS
hsa-miR-337-3p	CLIMB ($p = 0.007$), AMIR ($p = 0.038$), EPIC ($p = 0.044$)
hsa-miR-142-5p	CLIMB ($p = 0.049$), AMIR ($p = 0.023$)
hsa-miR-199a-5p	CLIMB ($p = 0.03$), AMIR ($p = 0.01$)
hsa-miR-330-3p	CLIMB ($p = 0.008$), AMIR ($p = 0.022$)
hsa-miR-194-5p	AMIR ($p = 0.034$), EPIC ($p = 0.01$)
hsa-miR-941	AMIR ($p = 0.039$), EPIC ($p = 0.05$)

Abbreviations: AMIR = AUBMC-Multiple Sclerosis Interdisciplinary Research cohort; CLIMB = Comprehensive Longitudinal Investigation of Multiple Sclerosis; EDSS = Expanded Disability Status Scale; EPIC = Expression/genomics, Proteomics, Imaging, and Clinical cohort; HC = healthy control; miRNA = microRNA; RR = relapsing-remitting MS; SP = secondary progressive MS; STOPMS II = Stockholm Prospective Assessment of Multiple Sclerosis.

differentially expressed in 2 of the 4 MS cohorts: hsa-let-7e-5p (AMIR and STOPMS II), hsa-let-7f-5p (CLIMB and STOPMS II), hsa-miR-486-5p (CLIMB and STOPMS II), and hsa-miR-30e-5p (CLIMB and EPIC) (table 4).

For the comparison of RR and HC, 2 miRNAs were differentially expressed in 2 of the 4 MS cohorts: hsa-let-7e-5p (AMIR and STOPMS II) and hsa-let-7f-5p (CLIMB and STOPMS II) (table 4). For the comparison of SP and HC, has-miR-320a, hsa-miR-320b, hsa-miR-320c, and hsa-miR-486-5p were differentially expressed in the CLIMB and STOPMS II cohorts (table 4).

When comparing the 4 cohorts in terms of association with the EDSS score, a statistically significant negative correlation with hsa-miR-337-3p was observed in 3 (CLIMB, AMIR and EPIC) of the cohorts. An additional 5 miRNAs (hsa-miR-142-5p, hsa-miR-199a-5p, hsa-miR-330-3p, hsa-miR-194-5p, and hsa-miR-941) showed a significant correlation in 2 of the cohorts (table 4).

When the prediction models from the CLIMB validation were applied to the transportability phase cohorts, the performance of the models was generally poor for most of the comparisons performed. The best performance was observed in the STOPMS II cohort for the SP vs HC comparisons (AUCROC = 0.73 for best subset model and AUCROC = 0.81 for random forests).

hsa-miR-337-3p as a marker for disability in MS

The expression of hsa-miR-337-3p negatively correlated with the EDSS in the discovery set of n = 85 (rs = −0.34; $p = 0.0002$) and the validation phases of n = 58 (rs = −0.30; $p = 0.02$).[9] These findings were further tested in another set of samples obtained from the CLIMB study (reproducibility phase) and in collaboration with 2 other international MS centers during the transportability phase (figure 1). Results from these validation studies confirmed that hsa-miR-337-3p is negatively correlated with the EDSS in 3 of 4 cohorts as summarized in figure, B.

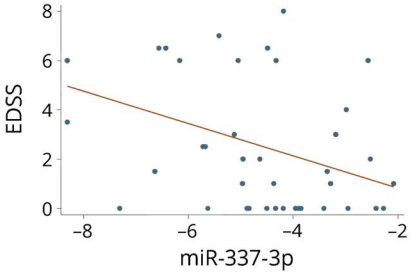
In addition, we found that in the CLIMB cohort SPMS patients have significantly decreased ($p = 0.01$) expression of hsa-miR-337-3p compared with the patients with RRMS (figure, C). The comparison between patients with SPMS and RRMS in the other 3 international cohorts showed a similar reduction in the expression of hsa-miR-337-3p in patients with SPMS compared with those with RRMS in 3 of the 4 cohorts (EPIC, AMIR, and CLIMB) (figure e-6, links.lww.com/NXI/A63).

Discussion

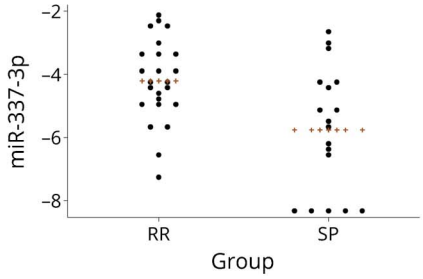
In this study, we tested the differential expression of serum miRNAs and the performance of prediction models in samples from untreated patients with MS. The study aimed at testing both reproducibility in CLIMB and transportability in multiple international centers. In our reproducibility phase cohort, we validated the differential expression of hsa-miR-484 in patients with MS as compared to HC (FDR < 0.05). In addition, we found an excellent performance of the prediction model including hsa-let-7c-5p and hsa-miR-452-5p discriminating MS from HC with an AUC of 0.85. hsa-miR-484 is a known factor in the regulation of neural progenitor cells and

Figure Correlation between the Kurtzke Expanded Disability Status Scale and the relative expression of hsa-miR-337-3p

A. CLIMB (rs = –0.41, $p = 0.007$)



C. CLIMB ($p = 0.010$)



B

	MS number	Mean MS	SD MS	Spearman correlation	*p* value
CLIMB	37	–4.41	1.33	–0.41	0.01
EPIC	26	–4.84	1.75	–0.35	0.04
STOPMS II	35	–5.56	1.51	0.02	0.87
AMIR	31	–4.56	1.37	–0.35	0.04

(A) Reproducibility phase: Spearman correlation between the Kurtzke EDSS and the relative expression of miR-337-3p in the CLIMB cohort. (B) Transportability phase: Spearman correlation between the EDSS and the relative expression of miR-337-3p across all MS centers. Number of patients with MS expressing hsa-miR-337-3p; mean MS = the mean expression value of hsa-miR-337-3p; SD MS = the SD from the mean of the expression value of hsa-miR-337-3p. (C) Differential relative expression of hsa-miR-337-3p in patients with RRMS compared with patients with SPMS in the CLIMB cohort, using LNA-based qPCR. Abbreviations: AMIR = AUBMC-Multiple Sclerosis Interdisciplinary Research; CLIMB = Comprehensive Longitudinal Investigation of Multiple Sclerosis; EDSS = Expanded Disability Status Scale; LNA = locked nucleic acid; RR = relapsing-remitting; SP = secondary progressive; STOPMS II = Stockholm Prospective Assessment of Multiple Sclerosis II cohort.

its decreased expression causes dysregulated synaptogenesis and is linked to neurodevelopmental conditions including epilepsy, autism, and hyperactivity[12]; hence, its increased expression in MS patients' sera might actually reflect the activation of neurogenesis pathways as part of an ongoing repair process.

Although no single miRNA was identified to be differentially expressed in all 4 cohorts when comparing SPMS vs HC, we found that the expression levels of hsa-miR-486-5p, hsa-miR-320a, hsa-miR-320b, and hsa-miR-320c were significantly upregulated in at least 2 independent international cohorts (table 4). Increased expression of hsa-miR-320a, hsa-miR-320b, and hsa-miR-320c also appears relevant to MS pathophysiology, and overexpression of miR-320 was found in MS lesions.[13] Multiple hsa-miR-320 targets previously reported could be involved in MS progression and in other diseases, such as cancer. Some of these targets include *CD71, MCL-1, MMP-9, NRP1, HDAC4, B-catenin*, and *MAPK*.[14–21] In this regard, the effect of hsa-miR-320 on MS pathogenesis is likely contextual, depending on the expression levels of the particular targets and the levels of this miRNA in specific cell types. One intriguing target for hsa-miR-320 is the proangiogenic *NRP1* in oral cancer.[17] *NRP1* is highly involved in regulatory T-cell development, and it has been reported that *NRP1* knockout mice exhibit a more Th17-like phenotype.[22] This would suggest a pathogenic role for hsa-miR-320 in MS pathophysiology. However, when considering the other potential targets, it appears that hsa-miR-320 could also serve a protective role in MS.[23–29] Of interest, differential expression of hsa-miR-320 is not unique to MS; we also found it to be significantly increased in the sera of patients with Alzheimer disease (AD) and asthma, whereas it was decreased in the sera of patients with amyotrophic lateral sclerosis (ALS) in comparison to HC samples (unpublished data and Raheja et al[30]).

We discovered that hsa-miR-337-3p and hsa-miR-199a-5p negatively correlated with patient disability (EDSS). The correlation of hsa-miR-199a-5p expression to the EDSS was observed in samples from 2 independent centers. The modest negative correlation of hsa-miR-337-3p with the EDSS was corroborated in 3 independent MS cohorts. In addition, the expression of hsa-miR-337-3p was found to be downregulated in patients with AD compared with healthy individuals, but was not differentially expressed in other diseases including asthma, rheumatoid arthritis (RA), and ALS (unpublished data). Our recent study on the correlation of MRI parameters to circulating miRNA expression also showed that the decreased expression of hsa-miR-337-3p is correlated with increased T1/T2 lesion load (Spearman correlation coefficient = −0.39; p = 0.012).[31] Thus, hsa-miR-337-3p is a potential biomarker candidate for MS disease progression. Of interest, it has been reported that hsa-miR-337-3p targets *RAP1A* in cancer.[32] As *RAP1A* is a well-established major component of integrin activation,[33] these findings points toward a potential role of hsa-miR-337-3p to serve as a biomarker for prediction of therapy response to natalizumab, an α4β1-integrin inhibitor.[34]

Some limitations encountered in this study include sample size, modest effect size of the reported findings, patient heterogeneity, and intercenter differences in patient selection and sample preparation and storage. Although we were able to confirm the transportability of several miRNAs in this international multicenter study population, we were also surprised that only few miRNAs were corroborated in all 4 centers. We believe that the inability to observe differences across all cohorts is likely attributed to these aforementioned limitations, in particular the low power of the study, especially considering the number of multiple comparisons. At the same time, we performed the analysis using all available samples rather than performing an a priori power calculation to determine the correct sample size. The heterogeneity of the population can be overcome in future studies by increasing the sample size across all comparison groups and using a standard procedure for patient's sample collection and storage. A sample size of 124 would be required to have 80% power to detect the all the differences observed in table 2 using a 2-sample t-test.

We were also unable to validate any differentially expressed miRNAs in the RRMS population across multiple centers. Notably, there is a higher variability in the EDSS scores of the patients with RRMS collected from multiple centers (table 1).

This is an attempt to examine the potential of miRNAs as biomarkers in an international multicenter setting for the diagnosis and correlation with disability in MS. It is also an attempt to build a prediction model in a validation phase after a training phase and a discovery phase. We found a promising candidate (hsa-miR-337-3p) to be used to identify a progression state that has great potential for translation to a clinical setting. Altogether, our work on circulating miRNA biomarkers is likely to help promote the development of novel tools for noninvasive diagnosis and therapeutic prognosis for MS.

Author contributions

R. Keren: drafting the manuscript for content, study concept and design, analysis and interpretation of data, and acquisition of data. B.C. Healy: drafting the manuscript for content, study concept, statistical analysis, and interpretation of data. A. Paul: analysis or interpretation of data, acquisition of data, and revising the manuscript for content. C. Diaz-Cruz: acquisition of data and revising the manuscript for content. M. A. Mazzola and R. Raheja: revising the manuscript for content. B. I. Glanz: design and patient selection. P. Kivisakk, T. Chitnis, M. Jagodic, F. Piehl, T. Olsson, M. Khademi, S. Hauser, J. Oksenberg, and S. J. Khoury: design, patient selection, and collection. H. L. Weiner: revising the manuscript for content, study concept and design, study supervision and coordination, and obtaining funding. R. Gandhi: drafting and revising the manuscript for content, study concept and design, analysis and interpretation of data, study supervision and coordination, and obtaining funding.

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