



Published in final edited form as:

*Muscle Nerve*. 2018 August ; 58(2): 261–269. doi:10.1002/mus.26106.

## Correlating serum microRNAs and clinical parameters in Amyotrophic lateral sclerosis

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### Abstract

**Introduction**—Amyotrophic lateral sclerosis (ALS) is a debilitating neurologic disorder with poor survival rates and no clear biomarkers for disease diagnosis and prognosis.

**Methods**—We compared serum miRNA expression from patients with ALS to healthy controls and patients with multiple sclerosis and Alzheimer's disease. We also correlated miRNA expression in cross-sectional and longitudinal cohorts of ALS patients with clinical parameters.

**Results**—We identified 7 microRNAs (miR-192-5p, miR-192-3p, miR-1, miR-133a-3p, miR-133b, miR-144-5p and miR-19a-3p) that were upregulated and 6 microRNAs (miR-320c, miR-320a, let-7d-3p, miR-425-5p, miR-320b and miR-139-5p) that were downregulated in ALS patients compared to healthy controls, Alzheimer's disease and multiple sclerosis patients.

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Ethical Publication Statement: We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Disclosures of conflict of interest: R. Raheja, K. Regev, M.A. Mazzola, V. Beynon, A. Paul report no disclosures. B.C. Healy served on the scientific advisory board for Biogen, Worldwide Medical Biostatistics, Multiple Sclerosis, served on the editorial board for *Statistical Methods in Medical Research*, received research support from Merck Serono, Genzyme, Novartis, Google Life Sciences, NIH, National Multiple Sclerosis Society. F. Glenn received research support from Sao Paulo Research Foundation—Brazil. C. Diaz-Cruz received research support from Merck Serono, Google Life Sciences. T. Gholipur received research support from Merck Serono, compensation as a reviewer from Boehringer Ingelheim, their spouse received compensation as a reviewer from Boehringer Ingelheim. B.I. Glanz received research support from Merck Serono SA, NIH, NMSS. P. Kivisakk received research support from EMD Serono, Sanofi Genzyme, Verily Life Sciences. T. Chitnis served on clinical trial advisory boards for Novartis Pharmaceuticals, Genzyme-Sanofi, consulted for Biogen Idec, Novartis, Genzyme-Sanofi, Genentech Roche, received research support from EMD Serono, Novartis, Biogen, Verily, National Multiple Sclerosis Society, the Peabody Foundation, the Consortium for MS Centers, Guthy-Jackson Charitable Foundation. H.L. Weiner served on the scientific advisory board for the Guthy-Jackson Charitable Foundation, Teva Pharmaceutical Industries Ltd., Biogen Idec, Novartis, Sanofi-Aventis, consulted for Therapix, Biogen, Novartis, Serono, Teva, Sanofi, National Multiple Sclerosis Society. J.D. Berry has consulted with Biogen, Denali Therapeutics and Neuraltus Pharmaceuticals, and has received research support from Voyager Therapeutics, GSK, Cytokinetics, Brainstorm Cell Therapeutics, and Novartis. R. Gandhi served on the editorial board for *Journal of Neurological Disorders & Stroke*, received research support from Novartis, Biogen, EMD Serono, Sanofi NIH, Nancy Davis Foundation, Harvard NeuroDiscovery Center, National Multiple Sclerosis Society.

Changes in 4 miRNAs (miR-136-3p, miR-30b-5p, miR-331-3p, and miR-496) correlated positively and change in 1 microRNA (miR-2110) correlated negatively with changes in clinical parameters in longitudinal analysis.

**Discussion**—Our findings identified serum microRNAs that can serve as biomarkers for ALS diagnosis and progression.

### Keywords

microRNA; serum; biomarkers; ALS; longitudinal analysis; disease comparisons

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## Introduction

Amyotrophic lateral sclerosis (ALS) is a debilitating neurodegenerative disorder characterized by the loss of motor neurons in the central nervous system (CNS)<sup>1</sup>. Currently, the two treatment options available, riluzole and edravone have modest effects on survival and rate of progression, respectively<sup>2,3,4</sup>. Both these treatments appear to be more effective when started early in the disease<sup>2,4</sup>. Hence, the current average diagnostic delay of approximately 12 months is potentially deleterious<sup>5</sup>. Furthermore, our lack of understanding of disease pathogenesis hinders target identification and novel ALS therapy development<sup>6</sup>. Biomarkers for use in early diagnosis, prognosis, or disease progression have the potential to hasten drug development and improve clinical trial design.

Although the majority of ALS cases occur sporadically (sALS) with incompletely understood etiology, approximately 10% are familial by pedigree (familial ALS; fALS)<sup>1</sup>. Many genes have now been correlated to ALS pathology – some of the most common and best understood include *SOD1*, *C9orf72*, *TARDBP* and *FUS*<sup>7,8</sup>. Although these genetic mutations are associated with fALS cases, they also occur in approximately 10% of sALS patients suggesting an underlying genetic component to the disease<sup>9,10</sup>.

Interestingly, two of the most commonly mutated genes in the disease, *TARDBP* and *FUS* are implicated in the biogenesis of microRNA (miRNA)<sup>11–13</sup>, thereby suggesting that dysregulation of miRNA expression might be a valuable tool to predict disease onset and progression. miRNAs are short (18–25 nucleotides) non-coding RNA that regulate gene expression by inhibiting mRNA translation or affecting mRNA degradation<sup>14</sup>. Recent studies suggest that several diseases including neurodegenerative disorders are associated with the altered expression of miRNAs<sup>15</sup>. Consistent with this, differentially expressed miRNAs have been identified in plasma, serum, spinal cord and cerebrospinal fluid (CSF) of ALS patients<sup>16</sup>. However, small patient cohorts and the absence of longitudinal evaluation have posed major limitations to the translation of these findings to clinical settings.

The utility of miRNAs for treatment and diagnosis is an active area of research. Several miRNA-based therapeutics are currently in phase I and II clinical trials for numerous disease indications including hepatitis C infections, type 2 diabetes, non-alcoholic fatty liver disease, cutaneous T cell lymphoma, and mesothelioma<sup>17–19</sup>. In addition, specific miRNAs are known to differentiate healthy versus disease states that has led to testing miRNAs as

diagnostic biomarkers in the clinic for cardiovascular, neurological, pulmonary disorders and various types of cancer<sup>19,20</sup>.

We aimed to identify miRNAs differentially expressed in serum samples from participants with ALS compared to both healthy participants and those with other neurologic diseases. We also studied the association between miRNA expression with disease progression. Finally, using available longitudinal data, we investigated whether changes in miRNA expression correlated with changes in clinical measures over time.

## Methods

### Participants

Patients included in the study met the El Escorial World Federation of Neurology Criteria<sup>21</sup> for clinically definite, probable, probable laboratory-supported or possible ALS. Participants were enrolled as a convenience sample from the Massachusetts General Hospital multidisciplinary ALS clinic and were assessed clinically at each visit. ALS disease duration was calculated from the day of onset of the first weakness or spasticity symptoms. The two parameters recorded at each visit were the revised ALS Functional Rating Scale (ALSFRS-R), and vital capacity (VC)<sup>22,23</sup>. Samples and clinical scores were collected longitudinally at approximately 3 to 6 month intervals. Of the 23 ALS patients enrolled, 3 were fALS and 20 were sALS). Two of the fALS patients had *C9orf72* repeat expansions and 1 was positive for an *SOD1* mutation. 17 patients (15 sALS and 2 fALS) were treated with riluzole. The remaining 6 patients were untreated. Table 1 includes the detailed information about ALS diagnosis, treatment regimen and the number of visits for each patient.

Healthy controls (HC) (30 participants, 9 males and 21 females, average age 43±12.2 years) were obtained from the Brigham PhenoGenetic cohort study and from healthy participants enrolled in the Comprehensive Longitudinal Investigation of Multiple sclerosis at Brigham and Women's Hospital and Partners MS Center (CLIMB, IRB 2013P002181/BWH). These subjects were recruited from the general population of Boston, are over 18 years of age, and have no chronic inflammatory, infectious or metabolic diseases<sup>24,25</sup>. Multiple sclerosis (MS) patient samples were obtained from the CLIMB study, and included relapsing-remitting (RRMS) (29 patients, 12 male and 17 female, average age 36±8.0 years ) secondary progressive (SPMS) (26 patients, 6 male and 20 female, average age 47±8.0 years), and primary progressive patients (PPMS) (18 patients, 9 male and 9 female, average age 51±8.0 years) who met the 2010 revisions to the McDonald criteria<sup>26</sup>. Alzheimer's disease (AD) patient samples (30 patients, 9 male and 21 female, average age 74±3.6 years) were obtained in collaboration with Dr. David Bennett, Rush Lab, Chicago. AD patients recruited for the study were cognitively non-impaired at the time of enrollment and ranged in age from 65 to 90<sup>24</sup>.

A signed informed consent was received from all of the participants. Secondary use approval for other disease samples was obtained (IRB 2013P002181/BWH).

## Samples and analysis

Blood samples were collected in glass red top serum vacutainer tubes without additives (BD Biosciences). Each sample was centrifuged at 2000 RPM for 10 minutes to separate serum and then stored at  $-80^{\circ}\text{C}$  until RNA extraction. Serum was frozen within 2 hours of the blood draw. Multiple freeze–thaw cycles were avoided by storing the samples in aliquots.

RNA was isolated using the miRcury kit (Exiqon, Waltham, MA) and converted to cDNA using a synthesis kit from Exiqon (Woburn, MA). Prepared cDNAs were stored at  $-20^{\circ}\text{C}$  until use. Locked Nucleic Acid (LNA)-SYBR- green-based real time PCR (RT-PCR) Custom made panel based on a prior study<sup>25</sup> containing 191 miRNAs, were used for profiling in the first set. Normalization was performed using the mean expression of miRNA with the best stability index, i.e., those that were stably expressed in our sample set. NormFinder software was used to calculate the stability index. We used four normalizing miRNAs (miR-15b-5p, miR-19a-3p, miR-126-3p and miR-425-5p). The following formula was used to calculate the normalized Cq values:

$$\text{Normalized Cq} = \text{average Cq} - \text{assay Cq}$$

## Statistical analysis

The expression of circulating miRNAs was compared between HCs, ALS, MS and AD patients.

For the ALS patients, the following three comparisons were made: a) baseline miRNA expression to baseline ALSFRS-R, baseline VC and disease duration, b) baseline miRNA expression to change in ALSFRS-R and VC measured over time during multiple patient visits, and c) changes in miRNA expression to changes in ALSFRS-R and VC measured over time.

For each miRNA, the number of subjects who had detectable expression level and the mean expression level was calculated for each group (ALS, HC, MS and AD). ALS patients were then compared to each of the three comparison groups separately using a Wilcoxon rank sum test. Familial and sporadic ALS patients were also compared to each other using a Wilcoxon rank sum test.

A Wilcoxon rank sum test was used so that participants with miRNA levels below the limit of detection (missing or undetected values) could contribute to the analysis. Undetected expression values were assigned a value 1 less than the minimum detectable miRNA level across all subjects. The summary statistics and p-values were calculated for the three group comparisons (Supplementary Tables 1–3) In addition to the individual miRNA comparisons, two logistic regression models were built with ALS vs. all other groups as the outcomes and combinations of miRNAs. The first logistic regression model included all miRNAs that were significantly upregulated in ALS patients in all three group comparisons (ALS vs. HC, ALS vs. MS, ALS vs. AD), and the second logistic regression model included all miRNAs that were significantly downregulated in ALS patients. Subsequent logistic regression models were generated by comparing these miRNAs expression specifically within the individual

groups (ALS vs. HC, ALS vs. MS and ALS vs. AD). The miRNAs chosen for inclusion in the multivariable logistic regression model were significantly associated with ALS in univariate analyses as shown in Supplementary Tables 1–3. The logistic regression coefficients and area under the ROC curve were calculated.

To investigate the clinical utility of miRNAs in ALS patients, we assessed both the cross-sectional and longitudinal associations between miRNAs and clinical outcomes. To assess the cross-sectional relationship between miRNAs and clinical measures, we estimated Spearman's correlation coefficient between the baseline measurement of each miRNA and the ALSFRS-R, VC and ALS disease duration. In addition to the cross-sectional comparison, the change in each of the clinical measures over the course of follow-up was calculated by fitting a linear regression with the ALSFRS-R or VC as the outcome and the time since the first measurement as the predictor. To assess whether baseline miRNA was predictive of change in clinical measures, we estimated Spearman's correlation coefficient between the baseline miRNA measurement and the slope in clinical measure. To assess whether change in miRNA was associated with change in clinical measures, we calculated the slope for each miRNA using a linear regression model and then estimated Spearman's correlation coefficient between the two slope estimates. If the miRNA and clinical measure changed in the same direction i.e. both increased or decreased, then they were positively correlated; however if one parameter decreased while the other increased they were negatively correlated. In addition to the main analyses, we also completed the same analyses adjusting for use of riluzole and the results were largely unchanged so only the unadjusted results are reported.

In all the comparisons, p-values were also adjusted for multiple comparisons using false discovery rate (FDR), and these values are provided in the supplementary material (Supplementary Tables 1–3). Statistical analysis was completed using the statistical packages R ([www.r-project.org](http://www.r-project.org)) and Stata/IC version 14 ([www.stata.com](http://www.stata.com)).

In all analyses, a p-value less than 0.05 was considered statistically significant. KEGG pathway analysis with miRNAs was carried out using DIANA miRPath<sup>27</sup>.

## Results

### Comparison of miRNAs expression between ALS patients, HC and other diseases

We found that 102 miRNAs were differentially expressed in at least one group comparison. Figure 1 shows the group comparison and the miRNAs that were specifically up- or downregulated in a single comparison and in comparison with one other group. We found 21 miRNAs that were only differentially expressed between ALS and HC, 11 miRNAs were only differentially expressed between patients with ALS and MS and 38 miRNAs were only differentially expressed between patients with ALS and AD. In addition to the miRNAs that were unique to a particular group comparison, 13 miRNAs were differentially expressed specifically in ALS patients compared to all other groups. Seven miRNAs were upregulated in ALS patients and 6 miRNAs were downregulated in ALS patients as compared to the three groups (Table 2). A logistic regression model was built with the 7 miRNAs that were upregulated in ALS in all comparisons. The estimated logistic regression coefficients are

provided in Table 2 and the ROC curve is shown in Figure 2a. The area under the ROC curve for this model was 0.86, showing clear discrimination between ALS and other groups. The 6 miRNAs that were downregulated in ALS in all comparisons were used to build a single model with the estimated logistic regression coefficients provided in Table 2, and the ROC curve is shown in Figure 2b. The area under the ROC curve for this model was 0.89. Additionally, logistic regression models were generated for specific group comparisons using the combination of 7 miRNAs that were upregulated in ALS in all three comparisons and the 6 miRNAs that were downregulated in ALS compared to all other group comparisons (Figure 2).

### Correlation of miRNAs with clinical parameters

The estimated Spearman's correlation coefficient comparing baseline miRNAs expression with baseline ALSFRS-R, VC and disease duration is provided in Supplementary Table 4, and a summary of the significant miRNAs is provided in Table 3. A positive correlation indicated that increased miRNA expression correlated with higher ALSFRS-R and VC values (Table 3). 4 miRNAs significantly correlated with ALSFRS-R score - 1 positively and 3 negatively (Table 3). 8 miRNAs negatively correlated with VC, and 6 positively correlated (Table 3). For disease duration, 7 miRNAs were negatively correlated (a decreased expression of these miRNAs is associated with longer disease duration), and 1 miRNA was positively correlated with disease duration (Table 3).

The estimated Spearman's correlation coefficients comparing baseline miRNAs and longitudinal change in ALSFRS-R and VC are provided in Supplementary Table 5. A positive correlation indicated that a high baseline expression is associated with a higher slope value or a lesser decrease with time, while a negative correlation indicated a high baseline miRNA expression is associated with a lower slope value or a greater decrease with time. 4 miRNAs correlated positively, and 2 miRNAs correlated negatively with ALSFRS-R change (Table 4). 8 miRNAs correlated positively and no miRNAs correlated negatively with changes in VC over time (Table 4).

Finally, we compared the change in miRNAs and the change in each clinical measure over time to assess whether miRNAs can be used as biomarkers of disease progression (Supplementary Table 5). The change in 4 miRNAs correlated positively with the change in both clinical measures and the change in miR-2110 correlated negatively with the change in both ALSFRS-R and VC (Table 5).

In a sub-analysis between sporadic and familial ALS patients, we found that the expression of miR-574-3p was significantly higher in sALS patients compared to fALS and miR-628-3p was significantly lower in sALS patients compared to fALS (Supplementary Table 6).

## Discussion

ALS biomarkers have the potential to help elucidate the pathogenesis of ALS, identify relevant pathways for drug development, provide biological patient strata, and after much validation, even act as surrogate endpoints in clinical trials. No single biomarker can fill all

these roles; in fact, each of these biomarker applications might require different characteristics. But as a group, miRNAs, which are highly varied but tightly controlled, broadly relevant and ubiquitously involved in cellular processes, could potentially serve many of these roles. Already, several studies have identified miRNAs in CSF, serum, plasma, leukocytes, spinal cord and skeletal muscle biopsies from ALS patients<sup>16</sup>. Using easily accessible serum samples, we have identified unique miRNA signatures that can distinguish participants with ALS from healthy participants and those with AD or MS. We also identified other miRNAs expression changes that correlate with disease state at baseline and over time and still others that predict rate of ALS progression as determined by changes in miRNA expression with changes in clinical measures such as ALSFRS-R score, VC and disease duration.

We observed some overlap with previously reported miRNAs (reviewed in<sup>16</sup>). For instance, miR-1, miR-133a-3p and miR-133b were previously found to be upregulated in plasma and serum samples from ALS patients<sup>28</sup>. In addition, miR-133b was also found to be downregulated in spinal cord biopsies<sup>29</sup>. Here, we report that miR-1, miR-133a-3p and miR-133b are upregulated in serum from ALS patients compared to other groups and consistent with this, their expression negatively correlates with VC. Another interesting miRNA, miR-451 has been reported to be downregulated in blood leukocytes from ALS patients<sup>30</sup> and we found that its expression positively correlates with VC indicating that lower expression is associated with worse disease. miR-142-3p was found to be upregulated in spinal cord tissue samples from ALS patients<sup>29,31</sup>. In our analysis, miR-142-3p negatively correlated with DD and low miR-142-3p expression correlated with rapid change in VC in our longitudinal studies.

In addition to intersecting with previously identified miRNAs, we also found striking physiological relevance for other miRNAs recognized in this study. Of the 7 upregulated miRNAs, miR-1, miR-133a-3p and miR-133b are myomiRs or muscle-specific microRNAs that are critical for the development and maintenance of skeletal muscles<sup>32,33</sup>. Previous studies have indicated an enhanced expression of Myogenic Differentiation 1 (MyoD1) and myogenin (MyoG) in ALS patients suggesting an active differentiation process in these patients<sup>34</sup>. Muscle atrophy is prevalent in ALS patients and an enhancement of homeostatic regulators of myogenesis might be a mechanism to cope with the muscular atrophy. miR-133b and miR-206 are encoded by a bicistronic transcript and have been studied in the G93A SOD1 mouse model. Upregulation of these miRNAs is associated with the onset of neurological symptoms in mice as a compensatory mechanism to regenerate neuromuscular synapses<sup>35</sup>. Our observation of enhanced expression of myomiRs in serum samples from ALS patients might be related to muscle atrophy and therefore serve as markers of disease onset and prognosis.

Among the downregulated miRNAs, miR-320a is known to target Aquaporins 1 and 4 (AQP1 and AQP4)<sup>36</sup>. The overexpression of AQP4 mostly in perivascular astrocytes has been observed in mouse and rat models of ALS and is associated with breakdown of the blood brain barrier (BBB)<sup>37</sup>. Impairment of the BBB though subtle, has been described in ALS patients<sup>38</sup>. Other members of the miR-320 family, miR-320b and miR-320c were also found to be downregulated in our samples. Interestingly, miR-320a, b and c are proposed

diagnostic markers for schizophrenia as their expression is downregulated in the peripheral blood of schizophrenia patients<sup>39</sup>. However, the molecular mechanism by which they might be contributing to schizophrenia remains to be investigated. An analysis of the common pathways regulated by miR-320a, miR-320b, miR-320c, miR-425-5p, let-7d-3p and miR-139-5p points to their role in the Hippo signaling pathway<sup>27</sup>. Mammalian sterile 20-like kinase 1 (MST1) is a protein kinase implicated in the Hippo pathway and its homozygous deletion delays the onset of ALS in mice<sup>40</sup>. One potential hypothesis for this effect might be that MST-1 induces neuronal cell death and muscle degeneration upon phosphorylation. While MST-1 is not a direct target of these miRNAs, they target upstream regulators such as large tumor suppressor kinase (*LATS1*, *LATS2*), and Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma (*YWHAG*) implicated in the Hippo pathway<sup>27,41</sup>.

Previous studies have shown that miR-574-3p is implicated in TDP-43 binding and is downregulated in lymphoblast cell lines in all patients except those with *SOD1* mutation<sup>12</sup>. Furthermore, both miR-628-3p and miR-574-3p are implicated in regulating DNA methyltransferases. There is growing evidence to indicate epigenetic changes in the promoter region of genes involved in processes such as calcium homeostasis, excitotoxicity and oxidative stress in patients with sALS<sup>42</sup>. The comparison of sporadic versus familial ALS was not the primary focus of the current investigation. Therefore, these studies warrant further investigation with larger patient groups to identify epigenetic signatures that might potentially distinguish groups of ALS patients.

Our longitudinal analysis suggests that miRNAs may be useful as prognostic biomarkers to provide information about expected rate of disease progression. We identified 5 miRNAs whose change in expression over time correlated with both changes in ALSFRS-R score and VC. Of these, the low expression of miR-136-3p, miR-30b-5p, miR-331-3p and miR-496 and high expression of miR-2110 correlated with more rapid disease progression. A KEGG pathway analysis of miR-136-3p, miR-30b-5p, miR-331-3p showed that these miRNAs are involved in the extracellular matrix (ECM) receptor pathway. Gene expression profile analysis in fibroblasts from patients with fALS indicated an enrichment in ECM receptor pathway and focal adhesion pathway molecules compared to healthy controls<sup>43</sup>. Accordingly, an upregulation of ECM-related genes was also observed in astrocytes from *SOD1* G93A mice, and these genes were downregulated in the spinal cord from presymptomatic *SOD1* G93A mice compared to WT mice. Some of the genes in this pathway that are upregulated in these disease models include Tumor necrosis factor (*TNF*), Endothelin1 (*END1*), and Angiotensin (*AGT*)<sup>44,45</sup>.

In a recent study comparing 12 different commercially available miRNA expression platforms for studying differential expression in serum, the LNA-based quantitative PCR platform from Exiqon, showed good reproducibility, accuracy and the highest specificity<sup>46</sup>. Therefore, we believe this is a robust system to measure the expression of miRNAs. However, due to the sample size and the exploratory nature of this study there were some limitations: 1) While serum samples from patients with MS and AD were used as controls to identify differentially expressing miRNAs in ALS, the use of serum samples from patients with other neurological disorders whose phenotypes overlap with that of ALS such as



inclusion body myositis and multifocal motor neuropathy will provide better differential diagnostic information, 2) not all miRNAs were expressed in every sample. In the group comparisons, the diverse age groups of participants in the AD group compared to others might account for this loss of miRNA expression and 3) only a subset of miRNAs remained significant after correcting for multiple comparisons using the false discovery rate (Supplementary Tables 1–3).

This study correlates changes in miRNA expression with changes in clinical parameters over time, providing insight into disease onset and progression. It provides the groundwork for confirmatory work in the future with a comprehensive validation in a larger cohort of patients, ideally with genomic information, collected in a multi-center study. This will enable the confirmation of these miRNAs as markers of diagnosis, disease prognosis as well as surrogate markers to serve as primary and secondary outcomes in clinical trials.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

This study was funded by NIH common funds via NCATS UH2/UH3 grant TR000890. We thank all the patients who consented to participate in this study.

## Abbreviations

<b>ALS</b>	Amyotrophic lateral sclerosis
<b>CNS</b>	Central Nervous System
<b>sALS</b>	Sporadic ALS
<b>fALS</b>	Familial ALS
<b>miRNA</b>	MicroRNA
<b>CSF</b>	Cerebrospinal fluid
<b>HC</b>	Healthy controls
<b>CLIMB</b>	Comprehensive Longitudinal Investigation of Multiple sclerosis at Brigham and Women's Hospital and Partners MS Center
<b>ALSFRS-R</b>	ALS Functional Rating Scale
<b>VC</b>	Vital capacity
<b>MyoD1</b>	Myogenic Differentiation 1
<b>MyoG</b>	Myogenin
<b>AQP</b>	Aquaporins

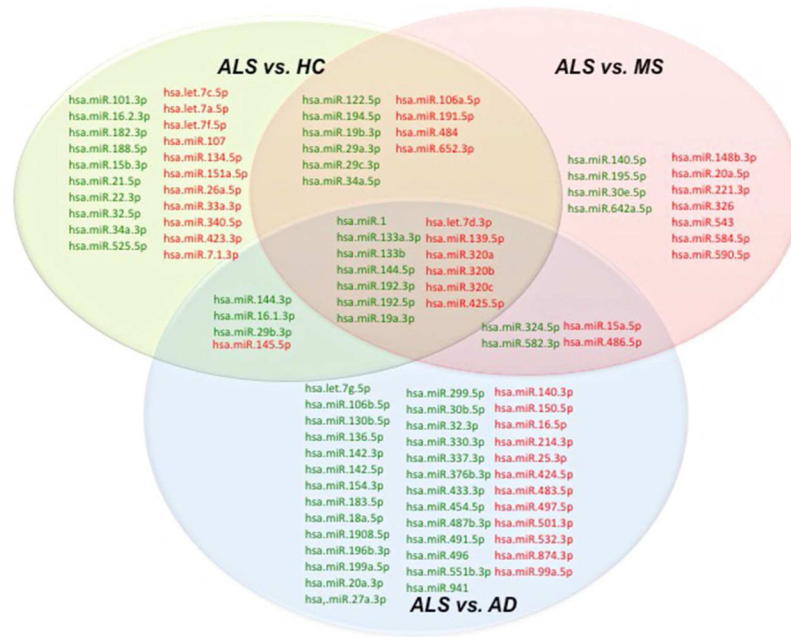
<b>BBB</b>	Blood brain barrier
<b>MST1</b>	Mammalian sterile 20-like kinase 1
<b>LATS1, LATS2</b>	Large tumor suppressor kinase
<b>YWHAG</b>	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein gamma
<b>ECM</b>	Extracellular matrix
<b>TNF</b>	Tumor necrosis factor
<b>END1</b>	Endothelin 1
<b>AGT</b>	Angiotensin

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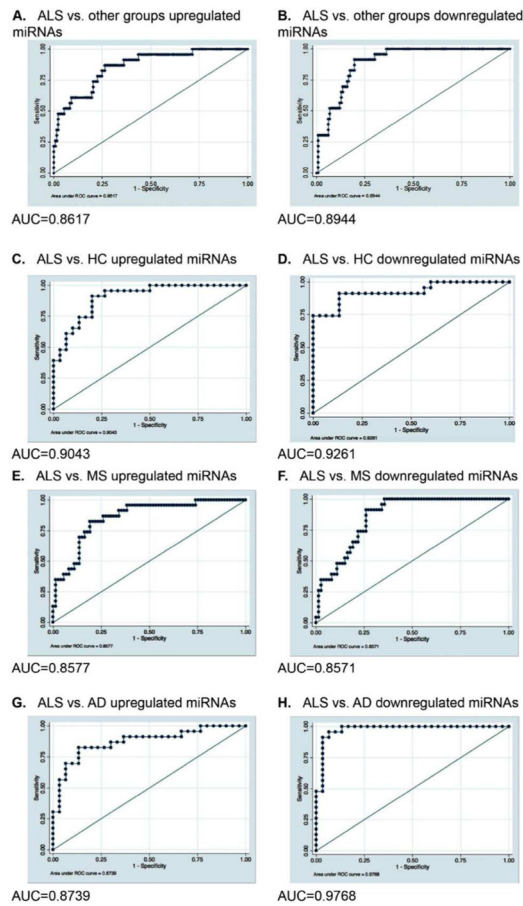
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**Figure 1. Venn diagram showing the serum miRNAs differentially expressed in ALS compared to Healthy controls (HC), Multiple sclerosis (MS) and Alzheimer’s disease (AD)**  
 Upregulated miRNAs are indicated in green and downregulated miRNAs are indicated in red.



**Figure 2. ROC curves for miRNAs differentially expressed in ALS versus other groups (HC,MS and AD)**

ROC curve for a combination of 7 miRNAs ( miR-1, miR-133a-3p, miR-133b, miR-144-5p, miR-192-3p, miR-195-5p and miR-19a-3p) upregulated in (A) ALS vs. all 3 groups, (C) ALS vs. HC, (E) ALS vs. MS, (G) ALS vs. AD . ROC curve for a combination of 6 miRNAs (let-7d-3p, miR-320a, miR-320b, miR-320c, miR-425-5p and miR-139-5p) downregulated in (B) ALS vs. all 3 groups, (D) ALS vs. HC, (F) ALS vs. MS, (H) ALS vs. AD ROC= Receiver Operating Characteristic, HC=healthy controls, ALS= Amyotrophic lateral sclerosis, MS= Multiple sclerosis, AD=Alzheimer's disease, AUC = Area under the curve.

**Table 1**

Detailed characteristics of ALS patients

El-Escorial criteria	ALS	Age	Gender	Treatment (Riluzole)	No. of visits
Probable Laboratory Supported	sporadic	70.8	Female	Yes	5
Definite	sporadic	43.1	Male	Yes	4
Definite	sporadic	44.2	Male	Yes	2
Probable	sporadic	65.3	Female	No	7
Possible	sporadic	57.4	Male	Yes	6
Probable Laboratory Supported	sporadic	75.8	Male	Yes	2
Probable	sporadic	71.4	Male	Yes	2
Probable Laboratory Supported	sporadic	53.3	Male	Yes	6
Possible	sporadic	54.5	Male	No	6
Probable Laboratory Supported	sporadic	68.2	Male	No	2
Probable Laboratory Supported	sporadic	57.8	Male	Yes	4
Definite	sporadic	57.9	Female	Yes	1
Probable Laboratory Supported	sporadic	49.3	Male	Yes	3
Probable	sporadic	57.9	Female	Yes	3
Probable	sporadic	40.7	Female	Yes	5
Probable	sporadic	53	Male	Yes	1
Definite	familial	54.8	Male	Yes	1
Definite	sporadic	50.7	Male	Yes	5
Probable	sporadic	48.5	Male	No	2
Definite	familial	50.8	Female	No	1
Definite	sporadic	55.4	Male	No	2
Probable	familial	41.6	Female	Yes	1
Probable	sporadic	54.4	Male	Yes	1

**Table 2**

Estimated coefficients from multivariable logistic regression model including all miRNAs upregulated and downregulated in ALS versus other diseases.

miRNA	Coefficient	SE	p-value	Lower limit of 95% CI	Upper limit of 95% CI
<b>Upregulated miRNAs</b>					
has-miR-1	0.219	0.249	0.378	-0.26854	0.70750
has-miR-133a-3p	0.183	0.3385	0.588	-0.47924	0.84573
has-miR-133b	0.392	0.355	0.27	-0.30404	1.08813
has-miR-144-5p	0.797	0.408	0.051	-0.00297	1.59599
has-miR-192-3p	0.320	0.162	0.048	0.002112	0.63876
has-miR-192-5p	0.600	0.380	0.115	-0.14526	1.34572
has-miR-19a-3p	2.092	0.790	0.008	0.54564	3.63861
<b>Downregulated miRNAs</b>					
hsa-let-7d-3p	-0.430	0.510	0.4	-1.42977	0.57114
hsa-mir-139-5p	-0.490	0.316	0.121	-1.11012	0.12977
hsa-miR-320a	1.652	1.156	0.153	-0.61316	3.91649
hsa-miR-320b	-0.271	1.054	0.797	-2.33706	1.79411
hsa-miR-320c	-3.718	1.397	0.008	-6.4574	-0.97951
hsa-miR-425-5p	-3.003	1.353	0.026	-5.65544	-0.35143

SE = Standard error, CI = Confidence interval



**Table 3**

Serum miRNAs correlating with baseline clinical parameters.

	miRNA	Correlation coefficient	p-value
ALSFRS-R	hsa-miR-32-5p	0.468	0.043
	hsa-miR-127-3p	-0.463	0.046
	hsa-miR-191-5p	-0.579	0.01
	hsa-miR-770-5p	-0.482	0.036
VC	hsa-miR-101-3p	0.606	0.006
	hsa-miR-15b-3p	0.528	0.020
	hsa-miR-19a-3p	0.635	0.003
	hsa-miR-361-3p	0.518	0.023
	hsa-miR-451a	0.622	0.004
	hsa-miR-92a-1-5p	0.494	0.032
	hsa-miR-1	-0.510	0.026
	hsa-miR-133a-3p	-0.729	0.0004
	hsa-miR-133b	-0.620	0.005
	hsa-miR-182-3p	-0.514	0.024
	hsa-miR-221-3p	-0.605	0.006
	hsa-miR-330-3p	-0.475	0.04
	hsa-miR-584-5p	-0.575	0.010
hsa-miR-589-3p	-0.492	0.033	
Disease duration	hsa-miR-9-3p	0.455	0.044
	hsa-miR-142-3p	-0.558	0.012
	hsa-miR-21-5p	-0.457	0.044
	hsa-miR-33a-5p	-0.450	0.048
	hsa-miR-34a-5p	-0.535	0.016
	hsa-miR-376b-3p	-0.464	0.039
	hsa-miR-491-5p	-0.473	0.035

ALSFRS-R = revised ALS Functional rating scale, VC = vital capacity

**Table 4**

Serum miRNAs correlating with clinical parameters in longitudinal analysis

Longitudinal analysis	miRNA	Correlation coefficient	p-value
Baseline miRNA / slope ALSFRS-R	hsa-miR-10a-3p	0.702	0.007
	hsa-miR-30e-3p	0.742	0.005
	hsa-miR-525-5p	0.613	0.026
	hsa-miR-598-3p	0.564	0.045
	hsa-miR-199a-5p	-0.610	0.030
	hsa-miR-27a-3p	-0.654	0.018
Baseline miRNA / slope VC	hsa-miR-133a-3p	0.725	0.007
	hsa-miR-133b	0.632	0.024
	hsa-miR-155-5p	0.560	0.05
	hsa-miR-182-3p	0.775	0.003
	hsa-miR-1913	0.558	0.05
	hsa-miR-22-5p	0.593	0.036
	hsa-miR-584-5p	0.610	0.030
	hsa-miR-99a-5p	0.731	0.006
Slope miRNA / Slope ALSFRS-R	hsa-miR-148b-3p	0.66	0.017
	hsa-miR-151a-5p	0.643	0.021
	hsa-miR-181c-3p	0.747	0.005
	hsa-miR-199a-3p	0.593	0.036
	hsa-miR-199a-5p	0.703	0.01
	hsa-miR-301b	0.588	0.038
	hsa-miR-324-5p	0.725	0.007
	hsa-miR-326	0.654	0.018
	hsa-miR-376b-3p	0.571	0.045
Slope miRNA / Slope VC	hsa-let-7c-5p	0.560	0.05
	hsa-miR-107	0.687	0.012
	hsa-miR-142-3p	0.610	0.030
	hsa-miR-154-3p	0.577	0.043
	hsa-miR-17-3p	0.118	0.700
	hsa-miR-181a-2-3p	0.648	0.02
	hsa-miR-423-3p	0.593	0.04
	hsa-miR-595	0.566	0.047
	hsa-miR-652-3p	0.604	0.032
	hsa-miR-320b	-0.610	0.030
	hsa-miR-483-5p	-0.577	0.043
	hsa-miR-532-3p	-0.626	0.025
	hsa-miR-589-3p	-0.599	0.034
	hsa-miR-874-3p	-0.626	0.025

ALSFRS-R = revised ALS Functional rating scale, VC = vital capacity

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**Table 5**

miRNAs correlating with changes in ALSFRS-R and VC

miRNA	Correlation coefficient ALSFRS-R score	p-value ALSFRS-R score	Correlation coefficient VC	p-value VC
hsa-miR-136-3p	0.637	0.022	0.687	0.012
hsa-miR-30b-5p	0.577	0.043	0.604	0.032
hsa-miR-331-3p	0.577	0.043	0.593	0.036
hsa-miR-496	0.687	0.012	0.571	0.045
hsa-miR-2110	-0.571	0.045	-0.687	0.012

ALSFRS-R = revised ALS Functional rating scale, VC = vital capacity

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