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miRNA extracted from extracellular vesicles is a robust biomarker of amyotrophic lateral sclerosis

Sandra Anne Banack [a], Rachael Anne Dunlop [a], Elijah W. Stommel [b], Paul Mehta [c], Paul Alan Cox [a,*]

[a] Brain Chemistry Labs, Jackson, WY, USA
[b] Department of Neurology, Dartmouth-Hitchcock Medical Center, Lebanon, NH, USA
[c] Centers for Disease Control and Prevention/Agency for Toxic Substances and Disease Registry, National ALS Registry (CDC/ATSDR), Atlanta, GA, USA

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ABSTRACT

Background and objectives: We examined miRNA biomarkers for ALS extracted from extracellular vesicles in blood samples using a large and diverse patient and control population. Different blood collection and storage protocols by different investigators could impact repeatability of miRNA analysis. We tested the hypotheses that miRNA extracted from extracellular vesicles using immunoaffinity purification techniques are robust and repeatable across investigators, laboratories and in a broad ALS population.

Methods: De-identified patient blood plasma samples obtained from the U.S. National ALS Biorepository were compared with plasma from non-ALS controls. Extracellular vesicles were extracted and isolated using L1CAM immunoaffinity purification. Total RNA was extracted, and miRNA quantified using qPCR following careful quality control measures. Gene fold expressions of eight miRNAs were compared using a Mann-Whitney two-tailed test.

Results: One hundred blinded, blood plasma samples were analyzed. Thirty-five men and 15 women with ALS were compared with controls consisting of 30 men and 20 women. None of the ALS patient cohort reported family members with ALS suggesting sporadic ALS. Five of the eight biomarkers previously published were found to significantly discriminate ALS patient samples from control samples.

Discussion: The methods used in this study provide a repeatable measure of miRNA biomarkers that statistically differentiate ALS patient samples from control samples. The broad inclusion criteria for both the ALS patient cohort and controls along with the collection of blood samples by different investigators suggest that these methods are robust and represent good candidates for further research and development aimed at clinical application.

1. Introduction

ALS is a progressive neurodegenerative disease with 24,000–31,000 identified American cases [1] and over 80,000 cases worldwide [2] at any one time. The mean life expectancy following diagnosis is 2–5 years [3]. Symptoms include progressive motor neuron degeneration resulting in weakness, spasticity, and muscle atrophy [4]. Diagnosis is based on clinical history, evidence of telltale neurological findings on exam including signs of progressive neurodegeneration of the upper and lower motor neurons, and electrodiagnostic findings of chronic and active denervation, while excluding other diseases with similar symptoms [4]. The disease is estimated to start years before a diagnosis is made and the lag time between symptom onset and diagnosis averages 10–16 months (range of 9 to 27 months) [4]. An incorrect diagnosis occurs in 13–68% of cases [4–6]. There is now compelling evidence for presymptomatic brain and spinal cord alterations long before symptom onset in both

Abbreviations: ALS, Amyotrophic Lateral Sclerosis; ALSFRS-r, amyotrophic lateral sclerosis functional rating scale-revised; ALS-FTD, amyotrophic lateral sclerosis-frontotemporal dementia; cDNA, complementary DNA; Cq, quantification cycle; EVs, extracellular vesicles; IRB, Internal review board; L1CAM, L1 cell adhesion molecule; miRNA, micro ribonucleic acid; NEE, neural enriched extracellular vesicles; QC, quality control; qPCR, Quantitative Polymerase Chain Reaction; SOD1, Superoxide dismutase 1.

* Corresponding author at: PO Box 3464, Brain Chemistry Labs, Jackson, WY, USA.
E-mail address: paul@ethnomedicine.org (P.A. Cox).

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SOD1 and C9orf72 mutation carriers [5–7]. Identifying pre-symptomatic biomarkers represents an important research goal [8].

Extracellular vesicles (EVs) are small, non-replicating particles released from cells that contain material from the source cell and are surrounded by lipid bilayer membranes. Cargo within EVs is considered promising material for prospective biomarkers because of the lipid bilayer membrane that protects the cargo from degradation [9]. Short non-coding RNA strands called micro RNA (miRNA) are frequently found in EVs and function to regulate post-transcriptional gene expression. In a replicated experiment using different patient cohorts, we previously identified eight miRNAs extracted from EVs (L1CAM enriched using immunoaffinity purification) that are diagnostic of early-stage ALS [10]. We report the results of a new experiment consisting of EVs extracted from 100 blood samples (50 ALS patients and 50 controls) designed to test the hypotheses that the eight miRNAs are robust biomarkers of ALS and can be replicated using the same techniques but less stringent plasma collection/storage parameters and a broader ALS population than the Phase II clinical trial patients (NCT03580616) we studied in the first experiment. The consistency of the results across different ALS patient populations provides evidence of the value of miRNA derived from L1CAM-enriched EVs for ALS diagnosis.

2. Methods

2.1. Blood plasma

Archived samples of blood plasma representing 50 patients with confirmed or probable ALS were obtained from the U.S. National ALS Biorepository, a component of the U.S. National ALS Registry maintained by the Centers for Disease Control and Prevention and the Agency for Toxic Substances and Disease Registry (www.cdc.gov/als). The blood plasma samples from the Biorepository were obtained by phlebotomists at different times and locations throughout the USA. The recommended protocol included blood collection in K2 EDTA tubes followed by 10× inversion. After collection the blood specimen was packed in Styrofoam-insulated shippers with properly conditioned reusable cold-packs to maintain a temperature between 4 and 8 °C for overnight shipping to a central location where they were processed and stored. In our original study, samples were spun-down in a refrigerated centrifuge within 1 h of collection and stored and transported at −80 °C [10]. Since no controls were available from the Biorepository, we used 50 samples of blood plasma from individuals not diagnosed with ALS (Innovative Research Inc., FDA Approval, #3003372368) as a control population. Sample use was reviewed by Advarra IRB Pro00053269 and since this study used de-identified participant data the IRB determined that this study "does not meet the DHHS definition of human subjects research under 45 CFR 46 and, therefore, does not require IRB oversight."

2.2. EV isolation

EVs were extracted as described previously (Section 2.3 EV Extraction in Banack et al. [10]) using polymer-based precipitation followed by immunoaffinity purification using the transmembrane protein L1CAM [mouse anti-human CD171 (L1 cell adhesion molecule (L1CAM) neural adhesion protein) monoclonal antibody (cat. no. eBIO5G3 (5G3), (13–1719-82), Biotin, eBioscience™ Antibodies, Thermo Fisher Scientific, Waltham, MA, USA] to generate a fraction we termed "neural-enriched extracellular vesicles" (NEE).

2.3. RNA extraction and quantification

2.3.1. Materials

The RNA extraction kit containing RNeasy MinElute Spin Columns was from Qiagen (ExoRNeasy Midi Kit #77144, Hilden, Germany). QIAzol lysis reagent 50 mL #79306, Buffer RPE (concentrate 55 mL) # 1018013, Buffer RWT (80 mL) #1067933, miRCURY RNA spike-in kit, for RT (containing UniSp2, 4, 5 and *C. elegans cel*-miR-39-3p) #339390, and the miRCURY LNA RT Kit containing UniSp6 #339340 were also from Qiagen. Chloroform ≥99%, stabilized, molecular biology grade #0219400225 was from MP Biomedicals (Irvine, CA, USA). Ethyl alcohol, pure 200 proof for molecular biology #E7023 (Lot #SHBJ8384), was from Sigma-Aldrich (St Louis, MO, USA).

2.3.2. RNA extraction

RNA was extracted from 100 μL NEE fractions using Part 2 of the Qiagen ExoRNeasy midi-kit (#77144), designed for isolating total RNA including retaining short RNAs, as described previously [11]. Briefly, 700 μL Qiagen QIAzol lysis reagent (#79306), containing 1 μL RNA extraction spike-in controls (UniSp2, UniSp4, and UniSp5, from the Qiagen miRCURY RNA spike-in kit, for RT #339390) was prepared for each NEE sample as described previously [11]. Total RNA was eluted into 15 μL nuclease-free water, placed on ice and cDNA prepared immediately. For alternatives to the Qiagen ExoRNeasy midi-kit, see Dunlop et al. [11].

2.3.3. cDNA synthesis

cDNA was prepared in 10 μL reactions, in duplicate, using the Qiagen miRCURY LNA RT Kit (#3393404) according to the manufacturer's instructions and containing 4 μL RNA and 1 μL of combined UniSp6 and *cel*-miR-39-3p spike-in reverse-transcription controls. UniSp6 (available in a lyophilized vial as part of the Qiagen miRCURY LNA RT Kit #339340), and *cel*-miR-39-3p (available as a lyophilized vial as part of the Qiagen miRCURY RNA spike-in kit, for RT #339390) were prepared as described previously [11].

The reaction conditions for each 10 μL sample were as follows: reverse transcription step, 42 °C, 60 mins; inactivation of reaction at 95 °C, 5 min, hold 4 °C, using a Thermo Scientific Arktik Thermal Cycler. The reactions were conducted in duplicate and pooled since the RT step is the most inconsistent of the entire qPCR process. We used 4 μL RNA rather than 2 μL RNA, as we previously determined this enables us to quantify low copy number miRNAs more accurately [11]. The pooled cDNA (20 μL) was aliquoted into 3 μL aliquots to avoid repeat freeze-thawing and stored at −20 °C until required for qPCR.

2.3.4. Quality control checks

2.3.4.1. Spike-in controls. To check that the RNA extraction and cDNA synthesis processes were consistent across samples, prior to conducting downstream qPCR, we quantified the spike-ins UniSp2, 4 and 5 (for RNA extraction control) and UniSp6 and *cel*-miR-39-3p (for cDNA synthesis/reverse transcription control) using qPCR and Qiagen miRCURY LNA miRNA SYBR Green PCR Assays (#339306, see Supplementary Table 1 for individual assay details). LNA miRNA SYBR PCR Assays come lyophilized and were re-suspended as follows: tubes were briefly centrifuged prior to opening, then 220 μL nuclease-free water was added, the tubes mixed by vortexing and spun down Assays were stored at −20 °C in aliquots to avoid freeze/thawing.

Quality control qPCR was conducted in 96-well plates in a final volume of 10 μL where 3 μL was cDNA (diluted 1/30 in nuclease-free water) and 7 μL was master mix (5 μL miRCURY LNA™ SYBR Green Master Mix, 1 μL re-suspended primer mix, and 1 μL nuclease-free water, per reaction) using the Bio-Rad CFX-96™ Real-time cycler C1000 Touch™ Thermal Cycler. The reaction conditions are described in Supplementary Table 3 and results for all 100 samples can be seen in Supplementary Fig. 1 to 9. For each target gene, duplicate no-template control wells were run simultaneously, and a melt curve was included for each primer pair.

2.3.4.2. Sample signal miRNA and hemolysis check. Since traditional methods for RNA quantitation, for example capillary gel electrophoresis

using a bioanalyzer, are not optimized for short RNA species such as miRNA, we measured the in-situ miRNA sample signal using qPCR. This was to ensure there was enough miRNA to proceed with downstream experiments as well as to check each sample for evidence of hemolysis during collection and processing. Five miRNAs were quantified: hsa-miR-142-3p, hsa-miR-451a, hsa-miR-23a-3p, hsa-miR-30c-5p, hsa-miR-103a-3p, and hsa-miR-191-5p (see Supplementary Table 1 for details). Hemolysis of erythrocytes during the processing of plasma can interfere with downstream results in qPCR and can be determined by measuring the ΔCq (hsa-miR-23a – hsa-miR-451a). If the ΔCq ≥ 7, then hemolysis is possible, and care should be taken deciding whether to use the sample in downstream processes (See Supplementary Data Fig. 1 to 9 for S1–100 sample signal and hemolysis data).

2.3.5. Quantitative real-time PCR of miRNA

qPCR for target and reference gene miRNAs (Supplementary Table 2) was conducted using Qiagen miRCURY LNA miRNA SYBR PCR Assays #339306 and the miRCURY LNA™ SYBR Green PCR Kit (g339347, Lot #76901890, SYBR Green Lot #N822.3) on the Bio-Rad CFX Opus 384 in 384-well plates according to the reaction conditions as detailed in Supplementary Table 3 and using the sample maximization method [12]. Briefly, cDNA was diluted 1/30 into nuclease-free water and 3 μL added to 7 μL master mix containing the miRNA primers of interest. All reactions were in a final volume of 10 μL. For each target gene, duplicate no-template controls were run on the same plate, as well as a melt-curve to determine the specificity of the amplification reaction.

2.3.6. Relative quantitation

For comparability with our previous study, we used the same reference genes as in Banack et al. [10] which were found to be stable using NormFinder [13]. The geometric mean was used for relative quantitation/normalization and a suitability check was performed following Vandesompele et al. [14]. Since the standard deviation of the ratio V ¾ was <0.15, we determined that no additional reference genes were needed [14]. Gene fold change was calculated using $2^{-(\Delta\Delta Cq)}$. Fold change was calculated as ALS mean/control mean and fold regulation was defined as equal to fold change when greater than one and as negative one divided by fold change when fold change was less than one.

2.3.7. Statistical analyses

The resulting gene fold change data were not found to conform to a normal distribution either using ΔΔCq values or log transformed data. For this reason, and to be consistent with the prior study, we used a two-tailed Mann-Whitney test to compare miRNA from ALS patients to the control population.

The following hypotheses were tested at the $p < 0.05$ level:

Ho=There is no difference in the medians of gene fold change $2^{-(\Delta\Delta Cq)}$ for miRNA from ALS and control patients.

H_1=There is a difference in the medians of gene fold change $2^{-(\Delta\Delta Cq)}$ for miRNA from ALS and control patients.

With large sample sizes, U* approximates the z values.

$U^* = \frac{U-mu}{\sigma u}$

$z = \frac{U-mu}{\sigma u}$where

$mu = \frac{n1n2}{2}$and

$\sigma u = \text{sqrt}\frac{n1n2(n1+n2+1)}{12.}$

3. Results

Target miRNAs: Five of the eight biomarkers we previously identified [10] were found to significantly discriminate ALS patient samples from control samples. Since data for gene fold expression $2^{(-\Delta\Delta Cq)}$ did not conform to a normal distribution, we used a two tailed Mann-Whitney test to determine statistical significance for the contrasting hypotheses. At $p < 0.05$, we were able to reject the null hypothesis that there was no difference in the medians of ALS versus control miRNA for hsa-miR-4454, hsa-miR-151a-5p, hsa-miR-146a-5p, hsa-miR-10b-5p and hsa-miR-29b-3p. Fold regulation was in the same direction as reported previously for all eight miRNAs (Table 1, Fig. 1, and Banack et al. [10]).

Clinically relevant data from the ALS patients was limited due to both the self-reported nature of the registry and constraints inherent in the biorepository collection. None of the patients reported parents, brothers, sisters, or children with ALS suggesting that the majority of these cases were sporadic ALS. The U.S. National ALS Registry allows patients to self-enroll and answer questionnaires but records age in decades to protect patient privacy which limits its utility for demographic comparison. Therefore, it was not possible to create an accurate age-matched control population, however, both groups had the same age inclusion criteria of >18 yrs. The ALS samples included 35 men and 15 women, and the control samples included 30 men and 20 women. The blood samples available for this study were generally not collected within a close time period relative to the reported ALSFRS-r scores (mean = 180 days, SD = 70) making it difficult to correlate clinical condition. Twenty-four patients had sequential ALSFRS-r scores (mean = 3.5 data points, SD = 1.6). Of those reporting sequential ALSFRS-r scores the period of reporting ranged from 44 to 1152 days (mean = 571, SD = 462). We plotted the slope of any patient that had recorded more than one ALSFRS-r score (mean = −0.02, SD = 0.03, Fig. 2). Despite the many missing values, self-reported nature of the values, and disparity in the length of time-points reported, these data suggest at least half the sample set patients lean towards a slow progression. No information concerning spinal or bulbar onset or comorbid conditions was collected by the U.S. National ALS registry.

3.1. Quality control

All samples passed QC tests including returning consistent raw Cqs for all spike-ins and sufficient quantity of sample miRNA to proceed with downstream processes (see Supplementary Data Fig. 1 to 9 for QC results for samples 1–100). We report four samples returned ΔCq ≥ 7 for hemolysis, (S14, ΔCq 7.46; S20, ΔCq 7.77; S35, ΔCq 7.05; S49, ΔCq 7.44) however, we determined to include these samples as the deviation from 7 was small (Supplementary Data Fig. 1 to 9).

4. Discussion

The need for better ALS diagnostic tools is readily apparent in the real-time challenges faced by ALS patients and clinicians. A misdiagnosis extracts a psychological and physical toll from patients who sometimes endure frequent referrals to specialists and unnecessary surgical procedures [4]. Research efforts to mitigate diagnosis delays through biomarker development are numerous. Putative biomarkers include spinal cord markers, protein aggregates, neurofilament subunits, electrical impedance myography, oxidative stress, neurotrophin receptor, proteins in peripheral blood mononuclear cells, inflammation, C-reactive protein, single photon emission computed tomography, and cortical hyperexcitability, to name a few [7,15–26]. The discovery of aberrant RNA metabolism and specifically miRNA dysregulation and altered miRNA biogenesis in ALS patients [17,27,28] supports the potential for finding useful diagnostic miRNA biomarkers for ALS [10,29–34].

Our hypothesis is that robust miRNA biomarkers are available for ALS diagnosis given reproducible techniques. There are concerns in the literature about the repeatability of RNA studies which stem from inadequate methods, experimental design, statistics, and documentation [35]. If a biomarker is to be developed for standard clinical use, it must be reproducible in different labs across time from samples collected by different investigators using different collection and storage protocols. The results of this study meet that criterion of differing collection and storage protocols in blood collection and qPCR analysis (the original qPCR in Banack et al. [10] was completed at Qiagen Genomics Services and the qPCR in the current study was performed at Brain Chemistry

Table 1
Potential miRNA biomarkers from L1CAM enriched EVs extractions in ALS patient blood plasma ($n = 50$) compared with control blood plasma (n = 50) as determined using qPCR analyses. *Z*-statistic is from a two-tailed Mann-Whitney test. Median values are reported as gene fold expression (2^ −ΔΔCq).

miRNA	Significance	Z-statistic	Median Control	Median ALS	Fold Regulation**	Regulation	Banack et al. (2020)
miR-4454	<0.01	−2.62	1.06	0.68	−2.7	down-regulated	down-regulated
miR-151a-5p	<0.05	−1.82	0.95	1.36	1.1	up-regulated	up-regulated
miR-146a-5p	<0.05	−1.94	1.06	1.29	1.1	up-regulated	up-regulated
miR-10b-5p	<0.05	−2.12	1.03	0.58	−3.2	down-regulated	down-regulated
miR-29b-3p	<0.05	−1.70	0.97	0.73	−1.5	down-regulated	down-regulated
miR-199a-3p	NS	−0.57	1.35	1.37	1.0	up-regulated	up-regulated
miR-199a-5p	NS	−0.83	1.39	1.71	1.1	up-regulated	up-regulated
miR-151a-3p	NS	−1.50	1.28	1.54	1.1	up-regulated	up-regulated

** Fold regulation was defined as equal to fold change (mean ALS / mean Control) when greater than one and as negative one divided by fold change when fold change was less than one.

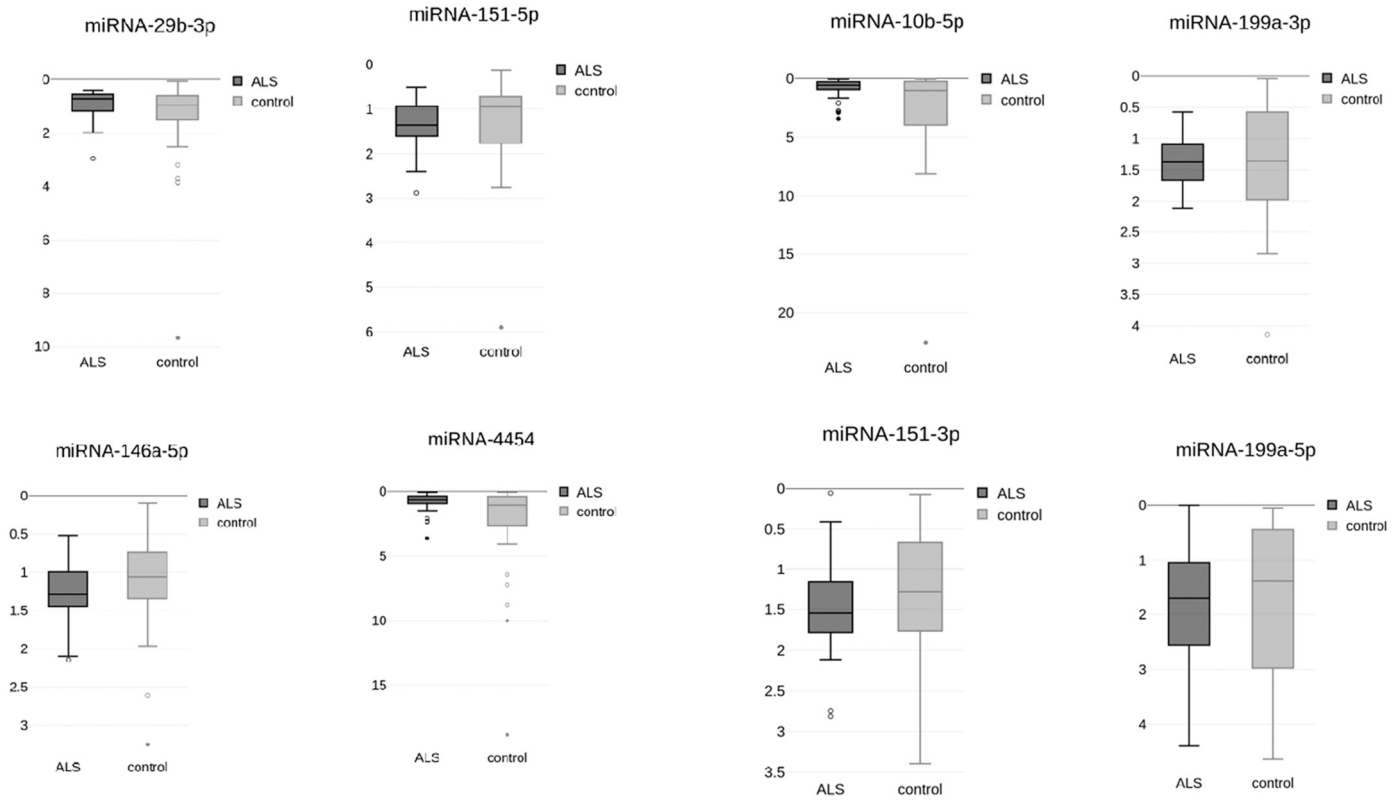

Fig. 1. Box-plot representation of variability in gene fold expression [$2^{-(\Delta\Delta Cq)}$] in five miRNA comparing ALS patient and healthy controls. A two-tailed Mann–Whitney *U* Test (non-parametric since data distribution plots did not conform to normal distributions) identified statistical differences ($p < 0.05$). Center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by circles.

Labs). Our methods followed protocols which have been rigorously tested and compared including extraction [36], spin-column [11], and PCR primers [37,38]. Internal quality control measures include blinding, spike-in controls for the RNA extraction and cDNA synthesis steps, determination of hemolysis, the inclusion of melt-curves and no-template controls, to ensure adequate target amplification in the absence of non-specific amplification and other artifacts and the use of the sample maximization method [12]. Separately, we have published a methods paper [11] titrating for the optimal amount of RNA to use in the cDNA synthesis reactions described here, to increase the likelihood that a robust Cq is generated from qPCR. We determined that 4 μL of RNA (as opposed to 2 μL as recommended by the manufacturer) was likely to return Cqs ≤ 35 in the miRNA targets we have chosen.

EV extraction polymer-based techniques are highly reproducible, and the L1CAM antibody affinity step enriches the sample in a predictable way. Tested in this study was possible variability in blood collection techniques and plasma storage conditions across labs. Equally of concern was the internal variability within ALS as a multifactorial disease with inherent clinicopathological heterogeneity [39,40]. Using a cross section of 50 ALS patients with a wide inclusion El Escorial criterion of definite or probable ALS allowed us to test the generality of these biomarkers. We found in samples from the U.S. National ALS Biorepository 5/8 of the original biomarkers collected within a Phase II clinical trial—with more stringent blood collection protocols and early-stage diagnosis determination by a single neurologist—as statistically significant. These five different miRNAs represent a robust ALS fingerprint worthy of further investigation for clinical use. Further tests involving ALS patients with known disease severity are required to evaluate the utility of using miRNA individually or in combination. Larger sample sizes and comparison with blood samples from ALS-mimic diseases, such as chronic inflammatory demyelinating polyneuropathy, ALS-FTD, or inclusion body myositis, are necessary to determine selectivity and sensitivity of these biomarkers.

Of particular interest, is the identification of miR-146a (up-

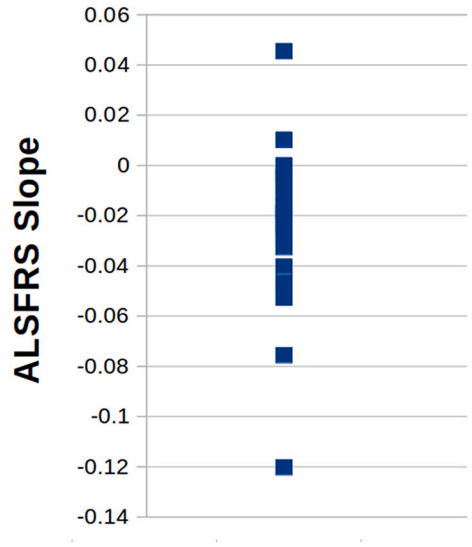

Fig. 2. ALSFRS-r slopes for 24 patients with multiple ALSFRS-r scores are plotted (mean = −0.02, SD = 0.03). Data were self-reported and entered by patients in the National ALS Registry. Mean number of data points per patient was 3.5 (SD = 1.6). Data represent a large variation in the number of days between the first and last score reported which affects the accuracy of the calculated slopes (range 44–1152 days, mean = 571, SD = 462).

regulated) and miR-4454 (down-regulated) which have been noted in other human ALS studies within circulating body fluids (i.e., not in EVs). miR-146a was also up-regulated in peripheral monocytes and CSF of sporadic ALS [41] and miR-4454 was down-regulated in plasma [42]. Furthermore, miR-146a was also noted as being expressed in the spinal cord of ALS patients but not in controls and is thought to regulate the low molecular weight neurofilament mRNA [17] which has itself been independently proposed as a possible ALS biomarker [19]. The presence of miR-146a has also been suggested to contribute to neuroinflammation [43] which may contribute to ALS pathology. As miRNA is being evaluated for use as biomarkers in many neurodegenerative diseases, it is interesting that there was not a significant difference in miR-4454 between Parkinson's disease patients and controls in plasma circulating RNA [44]. We consistently found differences between ALS and controls for miR-4454 which warrant further scrutiny.

The reason is unknown for a discrepancy in findings by other research groups of a down-regulation of miR-151a-5p circulating in blood within ALS patients [45] while we found miR-151a-5p is up-regulated in EVs enriched in L1CAM. Dobrowolny et al. [29] found miR-151a-5p up-regulated in the early stages of disease and down-regulated in the end stage disease. Data on the rate of decline in this study were limited by self-reporting parameters of the U.S. National ALS Biorepository. Further research comparing miR-151a-5p to disease stage and functional decline is warranted.

Similarly, we found miR-199a-3p consistently up-regulated while Saucier et al. [42] found it down-regulated in different fractions of biological fluids. This study replicating the earlier findings [10] found a similar up-regulation of miR-199a-3p but did not find a significant difference between ALS patients and control patients in this miRNA, suggesting that the difference between these two populations may not be sufficient to include this as an independent diagnostic biomarker. However, given the downregulation of both miR-199a-3p and miR-151a-5p seen in EVs from Parkinson's disease at specific disease stages [46], these two miRNAs might serve as important factors to separate ALS from PD given more research, and could shed light on pathophysiological mechanisms that are different between the two neurodegenerative diseases.

The other consistent and robust miRNA using these methods, miR-10b-5p and miR-29b-3p, should be examined further for clinical use. Since a combination of diagnostic blood-based biomarkers will likely be clinically useful, these findings are not mutually exclusive and could be combined with biomarkers identified by other researchers.

In our prior publication on ALS biomarkers [10] we suggested that immunoaffinity to the transmembrane protein, L1, which acts as a cell adhesion molecule, might permit a collection of neural-enriched EVs. This suggestion is well supported by findings of L1CAM expression on primary cortical neurons [47–49] and later by the demonstration of L1 expression on EVs collected within the 4–6 density gradient (DG) fraction in mature cortical neurons [50]. These findings contrast with a recent study [51] which found only minor overlap between the EV fraction of DG and the L1CAM immunoaffinity fraction. Norman et al. [51] concluded that L1CAM was not specific enough to be a neural marker. Our methods, whether representative of neural-enriched EVs or not, produce a repeatable pattern of ALS biomarkers that reliably distinguish ALS patients from controls making them potentially useful in the clinic, irrespective of origin.

Ideally, we would like to be able to correlate symptom onset, symptom duration, genetics, disease progression, and the prognostic and diagnostic potential of the miRNAs identified. The self-reported nature of the U.S. National ALS registry and the discordance between the sample collection times and reported ALSFRS-r scores in these samples were a constraint. Furthermore, we note the need to test these miRNAs against a cohort of ALS-mimics in order to establish the specificity of these markers to ALS. Given the results shown herein, we are now confident that the methods are robust and repeatable creating the background necessary to recommend further research in this area.

In conclusion, we have further replicated a prior study which identified eight miRNAs as candidiate biomarkers diagnostic for ALS using polymer-based precipitation for EV extraction and L1CAM for immunoaffinity purification. We found 5/8 of our original miRNAs were robust using a larger sample size (n = 50 ALS and n = 50 controls) but less stringent requirements for blood plasma collection/ storage, ALS diagnosis from multiple neurologists, and no criteria for the stage of disease. Further research is necessary to determine if all eight miRNAs may be useful specifically for early diagnoses and prove useful for predicting rate of progression.

Declaration of Competing Interest

The not-for-profit research institute Brain Chemistry Labs has applied for a patent on the use of this biomarker.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jns.2022.120396.

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