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(Article begins on next page)

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Identification of miRNAs as Potential Biomarkers in Cerebrospinal Fluid from Amyotrophic Lateral Sclerosis Patients

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Abstract

Amyotrophic lateral sclerosis (ALS) is a progressive, fatal neurodegenerative disorder. Since no diagnostic laboratory test exists, the identification of specific biomarkers could be fundamental in clinical practice. microRNAs (miRNAs) are considered promising biomarkers for neurodegenerative diseases. The aim of the study was to identify a CSF miRNA set that could differentiate ALS from non-

ALS condition. miRNA profiling in CSF from ALS patients ($n = 24$; eight with *C9orf72* expansion) and unaffected control subjects ($n = 24$) by quantitative reverse transcription PCR identified fourteen deregulated miRNAs. Validation experiments confirmed eight miRNAs as significantly deregulated in ALS. No significant differences were observed between ALS patients with or without *C9orf72* expansion. The receiver operator characteristic (ROC) curve analyses revealed the highest diagnostic accuracy for the upregulated miR181a-5p and the downregulated miR21-5p and miR15b-5p. The miR181a-5p/miR21-5p and miR181a-5p/miR15b-5p ratios detected ALS with 90 and 85 % sensitivity and 87 and 91 % specificity, respectively, confirming the application potential as disease biomarkers. These deregulated miRNAs are implicated in apoptotic way and provide insight into processes responsible for motor neuron degeneration.

Keywords

Amyotrophic lateral sclerosis
Cerebrospinal fluid
microRNA
C9orf72 expansion
Biomarkers

Michele Benigni and Claudia Ricci have contributed equally to this work.

Introduction

Amyotrophic lateral sclerosis (ALS) is an untreatable, fatal disease characterized by neurodegeneration involving primarily motor neurons in the motor cortex, brain stem and spinal cord. The selective loss of motor neurons causes the progressive wasting and weakness of limb, bulbar and respiratory muscles, paralysis and death, within 2–5 years of symptom onset (Robberecht and Philips 2013). Although most cases of ALS are reported as sporadic (90 %), about 10 % of cases have a family history of ALS (FALS) (Renton et al. 2014). Most commonly, familial ALS presents with a Mendelian dominant mode of inheritance and high

penetrance, although pedigrees with incomplete penetrance or recessive inheritance have been reported (Andersen and Al-Chalabi 2011). Currently, more than twenty-five genes and several additional chromosomal loci have been related to ALS, even though there is consensus about the causal role only for a subset of major genes as *C9orf72*, *SOD1*, *TARDBP*, and *FUS* (Marangi and Traynor 2015). Among these, the massive hexanucleotide-repeat expansion (GGGGCC) n, in the uncharacterized *C9orf72* gene, represents the most frequent genetic alteration in FALS (23–47 %) and SALS (4–21 %) identified to date (Millecamps et al. 2012).

Although the pathogenic mechanisms of neurodegeneration in ALS remain elusive, it is commonly accepted that multiple interactions among genetic components, environmental influences and epigenetic mechanisms contribute to disease development. It was recently observed that epigenetic mechanisms such as histone modifications, DNA methylation, RNA editing and posttranscriptional silencing mechanisms contribute to the development and course of ALS pathophysiology (Paez-Colasante et al. 2015). Modifications in RNA metabolism, including alterations in microRNA (miRNA) biogenesis, spliceosome integrity and RNA processing, are emerging as a novel common pathway responsible for motor neuron degeneration. Several apparently divergent biological effects can converge in this pathway, leading to a disturbance in protein expression and maturation, which contributes directly to motor neuron death (Droppelmann et al. 2014). In particular, increasing evidence underlines that miRNA deregulation is involved in ALS pathogenesis (Maciotta et al. 2013; Goodall et al. 2013; Eitan and Hornstein 2016). miRNAs are short non-coding RNA molecules (18–25 nucleotides) central to the epigenetic process that play an important role as endogenous regulators of gene expression. These small RNAs are capable of controlling gene expression either by repression of translation (Bartel 2009) or by mRNA degradation (Wahid et al. 2010). The same miRNA may regulate hundreds of target mRNAs and thus may affect complex gene expression networks. miRNA expression patterns can therefore be very informative. Moreover, miRNA molecules are present in human body fluids in a remarkably stable form (Mitchell et al. 2008; Gallego et al. 2012). Recent evidence indicates that these miRNAs can reveal changes in the

cells of origin, including neurons. In the case of CNS disorders, miRNAs detected in CSF can reflect brain physiological and pathological conditions and represent a more sensitive marker of changes than those present in blood or other body fluids (Rao et al. 2013; Roberts et al. 2015). For these reasons, in the last few years, miRNAs have aroused great interest as potential biomarkers in neurological disorders. Expression levels of miRNAs in CSF have been investigated in Multiple Sclerosis (MS) (Haghikia et al. 2012), Alzheimer's Disease (AD) (Cogswell et al. 2008; Müller et al. 2014; Burgos et al. 2014; Denk et al. 2015) and Parkinson's Disease (PD) (Burgos et al. 2014), with promising results. Specific biomarkers for ALS have not been identified yet, and the diagnosis of ALS is mostly based on electrophysiological examinations and clinical assessment with a history of symptom progression, with a considerable delay from symptom onset. The identification of specific biomarkers would allow an earlier and accurate diagnosis of ALS, with the opportunity to start an earlier treatment able to alter the disease course. Moreover, the finding of a deregulated miRNA expression in patients with ALS could provide important insights about the pathogenesis of the disease and eventually contribute to the development of potential future therapies.

To date, only two reports have been published on miRNAs in CSF from ALS patients using different approaches. In the first study, CSF samples from 22 ALS patients and 24 healthy controls were screened for a specific set of miRNAs binding TDP-43 (Freischmidt et al. 2013), identifying a subset of 5 deregulated miRNAs. In the second study, carried out on 10 ALS patients and 10 unaffected controls, the authors investigated the expression of one selected miRNA, previously found over-expressed in ALS blood leucocytes (De Felice et al. 2012, 2014). In the present study, we have performed the profiling of the miRNA population in CSF from ALS patients and unaffected control subjects, to identify a pattern of miRNAs that is specifically deregulated, and able to discriminate ALS from non-ALS. We profiled the expression of a panel of most abundantly expressed and best-characterized miRNAs by quantitative real-time PCR (qRT-PCR), a well-established method, which represents a highly sensitive and specific mean~~s~~ for the evaluation of miRNA profiles.

Materials and Methods

Patient Data and CSF Samples

CSF samples from 24 sporadic ALS patients were available for the present study. ALS diagnosis was performed according to World Federation of Neurology El Escorial Revised criteria, and patients with no evidence of a family history of the disease were classified as sporadic (Brooks et al. 2000). All patients were previously screened for mutations in the major genes associated with the disease.

Among the 24 patients included in the study, 8 were positive for *C9orf72* expansion. The unaffected control group consisted of 24 age- and sex-matched subjects who underwent lumbar puncture for neurological or microbiological diagnostic purposes and were negative for all the performed tests. Characteristics of ALS patients and controls are summarized in Table 1.

Table 1

Characteristics of ALS patients and controls

	Patients w/o mutation	***C9orf72* patients**	**Control subjects**
No of subjects	16	8	24
Gender	8M/8F	4M/4F	12M/12F
Age mean ± SD (years)	63.94 ± 8.45	60.50 ± 8.50	58.25 ± 5.04
Age at onset	59.0 ± 14.4	57.55 ± 12.6	
Bulbar onset	25.0 %	25.0 %	
Disease duration (months)	41.5 ± 20.5	47.0 ± 19.7	

The CSF, free of blood contamination, was centrifuged (1600×*g*, 4 °C, 15 min), frozen within 40 min of collection and stored at −80 °C until use. All procedures from withdrawal to storage of CSF samples were performed according to the Guidelines for CSF Biobanking for Biomarker Research (Teunissen et al. 2014).

miRNA profiling was performed in a total of six pooled CSF samples (total volume 200 µl), consisting of three pools derived from ALS patients and three pools from control subjects. Each pool was composed of an equal volume (25 µl) of four CSF samples from females and four CSF samples from males. miRNA validation experiments were carried out starting with 200 µl of CSF from each individual.

Written consent was obtained from each participant. This study was approved by the local ethics committee in accordance with the ethical standards of the Declaration of Helsinki and was carried out according to the international Good Laboratory Practice (GLP) and Good Clinical Practice (GCP) standards.

RNA Eextraction and Reverse Transcription

Total RNA was extracted employing the miRNeasy Mini kit (Qiagen, Hilden, Germany). Synthetic exogenous Ce-miR-39 (Cel-miR-39-3p) from *Cenorabditis Elegans* was added into each sample according to the manufacturer's recommendations. Purified RNA was stored at −80 °C in RNase-free water. Standard procedures were employed to ensure the quality and the reproducibility of the pre-analytical step.

Isolated RNA was used as starting material for reverse transcription (RT) employing the miScript II RT kit (Qiagen) that provides the polyadenylation of mature miRNAs and reverse transcription into cDNA using oligo-dT primers. In order to manage the quality of performed procedures, we used miScript miRNA QC PCR Array (Qiagen) that ensures the selection of only high-quality samples, employable in subsequent experiments, by testing the quality of RNA isolation and cDNA preparation.

miRNA Profiling by qPCR

cDNA prepared by RT reaction from RNA of pools was utilized as the template for real-time quantitative PCR (qPCR) analysis, performed in the 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA) using the human-miFinder 384HC miRNA PCR array (Qiagen), which profiles the expression of 372 most abundantly expressed and best-characterized miRNAs in miRBase

(www.mirbase.org). According to the manufacturer's recommendations, a final volume of 10 µl containing cDNA properly diluted and SYBR Green-based real-time PCR (Qiagen) was dispensed on human-miFinder PCR array.

Relative expression quantification was performed by the comparative cycle threshold (C_t) method ($2^{-\Delta\Delta Ct}$). Thus, the first step of qPCR analysis consisted of reference miRNA selection for normalization of miRNA expression. The miRNA608 and miR328-3p were selected by RefFinder free software (Xie et al. 2012) from a panel of eight potential endogenous reference miRNAs, since their expression showed high stability and reproducibility across different samples and experiments. The miR-39-3p of *Caenorhabditis elegans* (Cel-miR-39-3p) was also employed as exogenous control for the normalization. The control group was used as the calibrator in the calculation of fold change. miRNAs with a fold change ≤-1.5 were considered downregulated and miRNAs with a fold change ≥1.5 were considered upregulated.

Free software miScript miRNA PCR Array Data Analysis (Qiagen), available at http://pcrdataanalysis.sabiosciences.com/mirna, was employed for the analysis of qPCR results of miRNA profiling experiments. Only miRNAs with C_t <35 and with a high efficiency amplification plot were taken into consideration for subsequent analysis. The ALS patients and control groups were compared using Student *t* test, and the cut off for the *p* value was set at 0.05.

miRNA Validation

Validation experiments of miRNAs selected in the profiling were performed in triplicate by SYBR Green-based real-time PCR analysing individually CSF sample obtained from each subject. The Cel-miR-39-3p and both endogenous miR608 and miR328-3p were used in the validation experiments as reference miRNAs to normalize miRNA expression values. Relative quantification was carried out using Data Assist v3.0 (Thermo Fisher Scientific). miRNAs were considered downregulated for fold change values ≤-1.5 and upregulated for values ≥1.5. Similarly to the miRNA profiling, the miRNA molecules with C_t >35 were excluded from subsequent data analyses.

Statistical Analysis for miRNA Validation

All statistical analyses were performed with SPSS (Version 18; SPSS Inc, Chicago, IL, USA) and GraphPad Prism (Version 5.0; GraphPad Software Inc, San Diego, CA, USA). Normality of data was assessed by the Shapiro–Wilk test. Statistical differences were verified by Student's unpaired two-tailed *t* test in the case of normal distributions, or two-tailed Mann–Whitney *U* test in the case of non-normal distributions. Analysis was performed for every miRNA, and ratios between different miRNAs were examined to identify any specific characteristics in ALS. The ratio between miRNAs was calculated as $2^{-\Delta Ct}$ ($\Delta C_t = C_t$ of upregulated miRNA − C_t of downregulated miRNA), as described by Sheinerman et al. (2012).

Receiver operating characteristic (ROC) curves were plotted to evaluate the power of miRNAs (singles or in combination) to differentiate ALS patients from controls.

Differences among groups of patients, stratified based on *C9orf72* expansion and clinical features (age, gender, site of onset, disease duration) were evaluated by Student's unpaired two-tailed *t* test or two-tailed Mann–Whitney *U* test in the case of normal distributions or non-normal distributions, respectively. Spearman's rho (*r*) was calculated to find correlation between miRNA expression level and age at onset. Association of each single miRNA expression level with disease duration was estimated using the Kaplan–Meier method and compared by the log-rank. Analysis was performed considering only deceased patients and considering both living and deceased individuals, using a censored approach. Patients were divided into high miRNA expression group (miRNA levels greater than the median) or low miRNA expression group (miRNA levels less than the median). *p* values smaller than 0.05 were considered statistically significant.

Results

miRNA Profiling and Identification of Differentially Expressed miRNAs

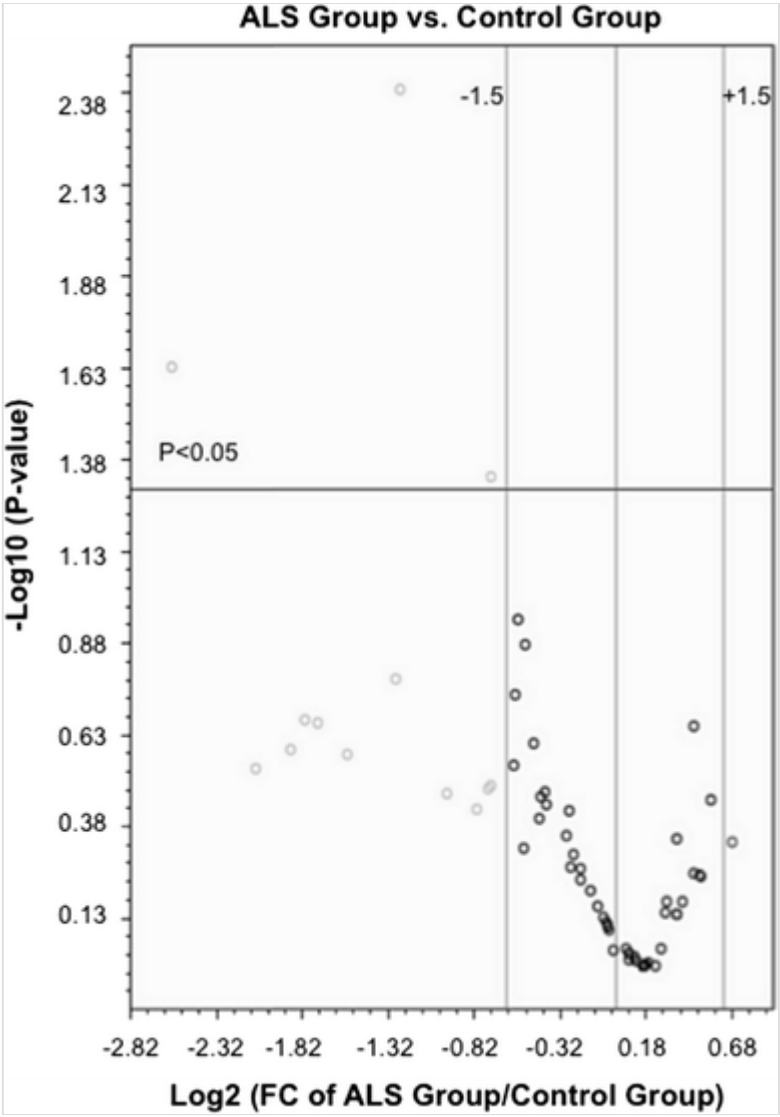
The CSF profiling permitted the amplification by real-time PCR of 35 (9.4 %) and 39 (10.4 %) miRNAs in CSF pools from ALS patients and

control subjects, respectively, for a total of 42 out of 372 (11 %) miRNAs positively detected in our sample cohort. No detectable traces or traces with $C_t > 35$ were identified for 330 miRNA molecules. Furthermore, our results showed an overall downregulation of miRNAs in CSF from ALS patients compared to controls; indeed, the majority of deregulated miRNAs were downregulated.

From miRNA profiling analysis, we selected thirteen downregulated miRNAs (let7a-5p, let7b-5p, let7f-5p, miR15b-5p, miR21-5p, miR122-3p, miR127-3p, miR148a-3p, miR150-5p, miR183-3p, miR195a-5p, miR204-5p, miR373-5p) and one upregulated miRNA (miR181a-5p). Among these deregulated miRNAs, three (let7-a, miR195a-5p and miR21-5p) were significantly downregulated with a *p* value of 0.0023, 0.039 and 0.030, respectively. The changes in the other eleven miRNAs did not reach statistical significance even though they were downregulated with a fold change ≥1.5. The results of miRNA profiling were graphed in the *Volcano Plot* reported in Fig. 1.

Fig. 1

Volcano Plot of $\log_2$ of the fold change for each miRNA and its *p* value. The *p* value is calculated based on the Student's *t* test of the replicate $2^{-\Delta Ct}$ values for each miRNA in the control group and patient group. The *central vertical line* in the graph indicates a fold change of 1. Vertical sliders indicate miRNAs as either up or down regulated with a fold change > 1.5. The spots beyond *right vertical slider* indicate the upregulated miRNA, and the spots beyond the *left slider* indicate the downregulated miRNAs. The *horizontal line*, parallel to the *x* axis, indicates the desired threshold for the *p* value of the Student's *t* test, defined <0.05. The Volcano Plot was generated by the software miScript miRNA PCR Array Data Analysis (Qiagen)

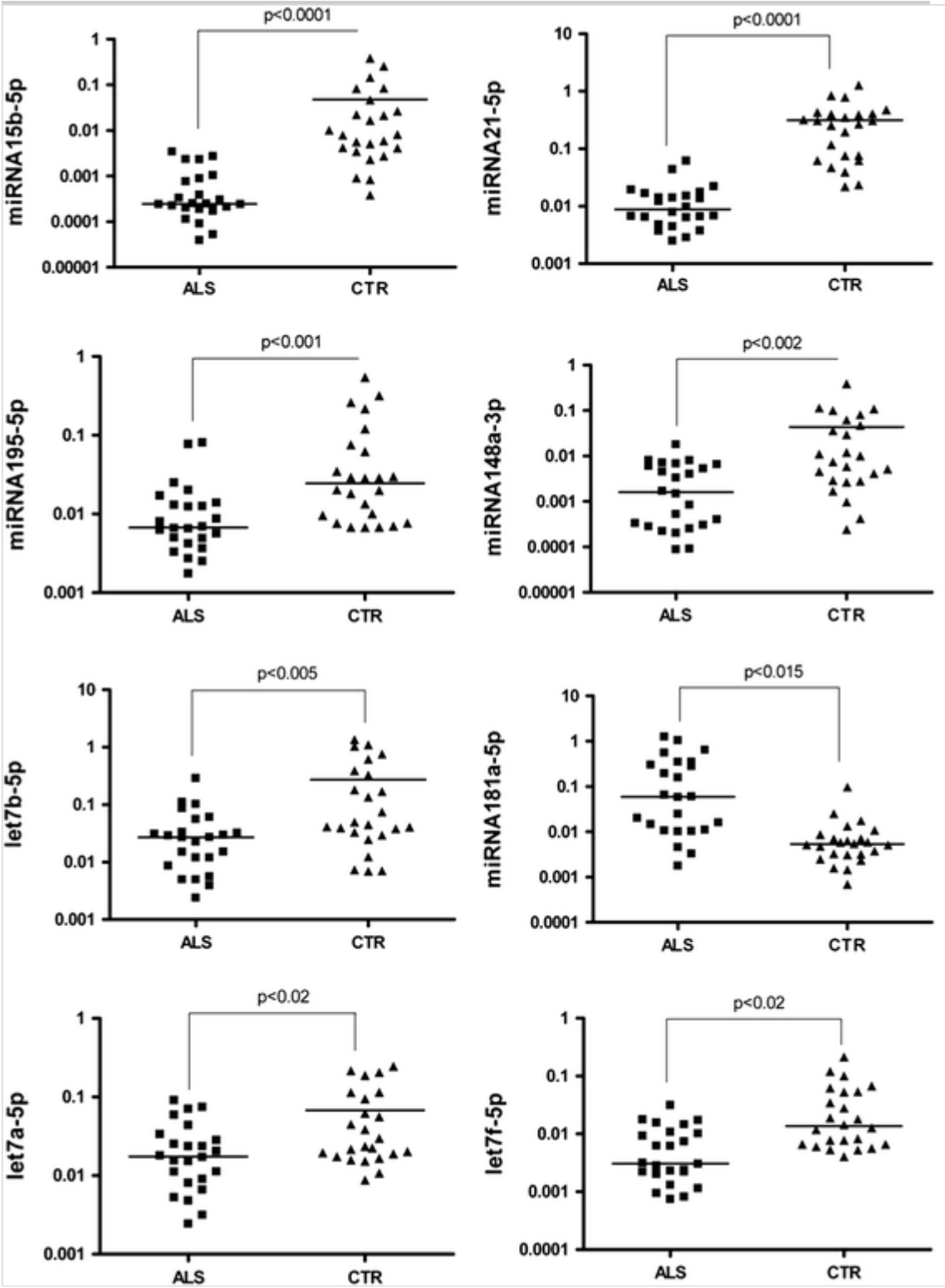


Expression profiles of selected CSF miRNAs

The fourteen deregulated miRNAs were validated in CSF samples obtained from each subject by qPCR. The deregulation observed in the profiling was confirmed, with an overall miRNA downregulation and only miR181a-5p upregulated. Among the fourteen selected molecules, eight miRNAs were significantly deregulated in ALS patients compared to controls (Fig. 2). In particular, changes in miR21-5p, miR195-5p and let7a-5p, which were significantly downregulated in miRNA profiling, were confirmed in the validation experiments, and a significant downregulation was also reported for miR148-3p, miR15b-5p, let7b-5p and let7f-5p. The miRNA181a-5p was confirmed as upregulated in CSF from ALS patients. No significant difference was evidenced for the other six miRNAs included in the validation experiments.

Fig. 2

Scatter graphs of results of CSF validation experiments for the eight significantly deregulated miRNAs. *p* values were calculated using two-tailed Mann–Whitney *U* test. Relative expression of deregulated miRNAs in CSF from ALS patients and controls normalized by $2^{-\Delta Ct}$ is graphed ($\log_{10}$ scale on the *y* axis), and medians are indicated as *horizontal lines*. *ALS* Amyotrophic Lateral Sclerosis patients, *CTR* control subjects



The levels of the eight deregulated miRNAs were analysed in the ALS patient group to evaluate possible correlations with *C9orf72* expansion and clinical variables. The *C9orf72* repeat expansion, which represents the most frequent genetic alteration in ALS, was present in 8 out of the 24 patients included in the study. No significant differences in miRNA expression levels were observed between ALS patients with or without the expansion. Association of miRNA levels with gender, site of disease onset (spinal versus bulbar) and age at disease onset was also evaluated. No significant associations were found with the ALS clinical variables examined. The results are summarized in Table 2. Survival analysis did not reveal any association with the disease duration, using a censored approach, and considering only deceased individuals (data not shown). A shorter disease duration was correlated with the contemporary presence of low miR15b-5p and miR21-5p expression levels and high miR181a-5p expression, even though the correlation was not statistically significant ($p = 0.064$).

Table 2

Correlations between miRNAs level and *C9orf72* expansion, and ALS clinical variables

miRNA	*C9orf72**	Disease duration (censored)	Site of onset	Gender	Age at onset
let7a-5p	0.750	0.240	0.266	0.436	0.769
let7b-5p	0.750	0.379	0.267	0.631	0.384
let7f-5p	0.750	0.253	0.349	0.971	0.658
miR15b-5p	1.000	0.070	0.497	0.684	0.270
miR21-5p	0.437	0.515	0.197	0.481	0.156
miR148a-3p	0.617	0.242	0.230	0.052	0.076
miR181a-5p	1.000	0.542	0.933	0.393	0.137
	0.211	0.745	0.211	0.796	0.286

* Patients were dichotomized in carriers and non-carriers of the *C9orf72* expansion

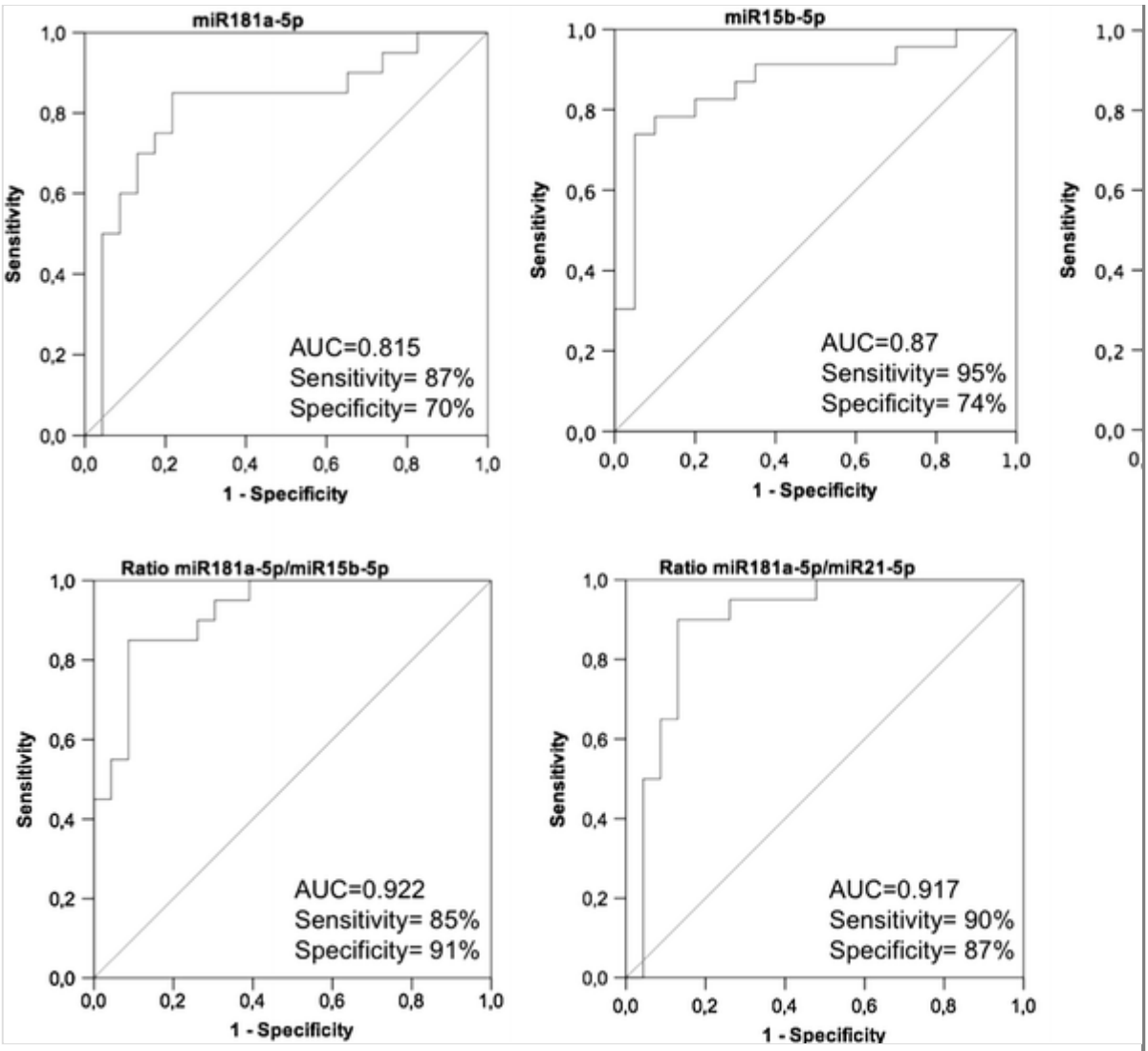
For each column *p* values are reported

miRNA	*C9orf72**	Disease duration (censored)	Site of onset	Gender	Age at onset
miR195-5p					
* Patients were dichotomized in carriers and non-carriers of the *C9orf72* expansion					
For each column *p* values are reported					

The receiver operator characteristic (ROC) curve analyses revealed that all the eight significant miRNAs could be potential biomarkers for ALS diagnosis. However, among these miRNAs, the upregulated miR181a-5p and the downregulated miR21-5p and miR15b-5p showed the highest diagnostic accuracy (Fig. 3). The miR181a-5p levels gave an area under the ROC curve (AUC) of 0.815 (95 % CI 0.677–0.953). At the cutoff value of 6.16, the optimal sensitivity and specificity were 87 and 70 %, respectively. The downregulated miR21-5p and miR15b-5p gave areas under the ROC of 0.878 (95 % CI 0.761–0.996) and 0.87 (95 % CI 0.757–0.982), respectively. At the cutoff value of 4.01 for miR21-5p, the optimal sensitivity and specificity were 95 and 74 %, respectively, and at the cutoff value of 8.17 for miR15b-5p the optimal sensitivity and specificity were 95 and 74 %, respectively.

Fig. 3

Receiver operating characteristic (ROC) curve for the upregulated miRNA (miR181a-5p) and the two-downregulated miRNAs (miR15b-5p and miR21-5p), which showed the highest significant differences in validation phase for discriminating ALS patients from controls. For miR181a-5p, the normalized expression level ($2^{-\Delta\Delta Ct}$) was selected as test variable and for the miR21-5p and miR15b-5p the logarithm of the normalized expression level. In the case of analyses in combination, ROC curve was obtained for the ratios between miRNAs, calculated as $2^{-\Delta Ct}$ ($\Delta C_t = C_t$ miR181a-5p − C_t miR21-5p and C_t miR181a-5p − C_t miR15b-5p). AUC (area under the ROC curve), sensitivity and specificity are reported for each ROC curve



The three miRNAs with the highest diagnostic accuracy, revealed by ROC curve, were analysed in combination. Values obtained from ratios between the upregulated miRNA and the two-downregulated miRNAs were examined comparing patients and controls. A significant deregulation was reported for the analyses of both miR181a-5p/miR21-5p ($p < 0.0001$) and miR181a-5p/miR15b-5p ($p < 0.0001$). The ROC curve analysis of miR181a-5p/miR21-5p ratio showed an area under the curve of 0.917 (95 % CI 0.836–0.999). At the cutoff value of 0.45, the optimal sensitivity and specificity were 90 and 87 %, respectively. The miR181a-5p/miR15b-5p ratio gave an area under the ROC of 0.922 (95 % CI 0.844–0.996). The optimal sensitivity and specificity were 85 and 91 % respectively, at the cutoff value of 29.8 (Fig. 3).

Discussion

This paper provides the first description of CSF miRNA profiling in ALS identifying a pattern of miRNAs specifically deregulated to use as biomarkers for ALS. The miRNA profiling, defined as the measurement of the relative abundance of a cohort of miRNAs detectable in cell-free (Pritchard et al. 2012), is the more promising approach for identification of miRNA biomarkers. We profiled the expression of a panel of 372 miRNAs representing the most abundantly expressed and best-characterized miRNAs in miRNA-databases. A total of 48 CSF samples (24 from sporadic ALS patients and 24 controls) were analysed applying qRT-PCR in two phases: initial screening by miRNA-array and subsequent validation of selected molecules. Among the various techniques for miRNA profiling, we chose the quantitative reverse transcription PCR (qRT-PCR) since it is the most specific and sensitive established (Pritchard et al. 2012). This technique provides the immediate availability of quantitative data using nanograms of RNA and gives the possibility to study a consistent number of specific miRNAs with well-known mRNA targets and molecular functions (Pritchard et al. 2012; Pacifici et al. 2014). As in several miRNA profiling studies, the initial screening was performed in pooled samples. This approach, using a sensitive assay such as qRT-PCR, offers a competitive and cost-effective tool for identification of ALS miRNAs.

The profiling allowed the detection of 42 out of 372 miRNAs. The percentage of miRNAs detected in our study (11 %) was congruent with that recently reported by Denk et al. (2015) (15 %) who used qRT-PCR to profile 384 different miRNAs in CSF from AD patients.

A total of fourteen deregulated miRNAs was selected from the profiling: thirteen that were downregulated (let7a-5p, let7b-5p, let7f-5p, miR15b-5p, miR21-5p, miR122-3p, miR127-3p, miR148a-3p, miR150-5p, miR183-3p, miR195a-5p, miR204-5p, miR373-5p) and one upregulated (miR181a-5p). We observed an overall downregulation of miRNAs in ALS samples, consistent with other studies reporting that the majority of deregulated miRNAs in tissues from ALS patients and ALS models are downregulated (Paez-Colasante et al. 2015).

In order to confirm these results, the expression levels of selected miRNAs were analysed in each single sample included in the pool,

using qRT-PCR. A group of 8 out of the 12 miRNAs selected by miRNA profiling were confirmed as significantly deregulated: seven were downregulated (let7a-5p, let7b-5p, let7f-5p, miR15b-5p, miR21-5p, miR148a-3p, miR195a-5p) and one upregulated (miR181a-5p). We observed an interesting overlapping of six deregulated miRNAs in our dataset (miR181a-5p, miR21-5p, miR148a-3p and let7a-5p, let7b-5p and let7f-5p) with those described by Burgos et al. (2013), who listed the 50 most abundant miRNAs detectable in CSF. This observation suggests that the majority of miRNAs selected in this study should be specific for nervous tissue and have a brain origin.

The selected miRNAs have not been identified as significantly deregulated in the two previously published studies (Freischmidt et al. 2013; De Felice et al. 2014). A possible explanation for this discrepancy could be the different strategies used to identify the deregulated miRNAs: the use of a set of TDP-43 binding miRNAs (Freischmidt et al. 2013), or the analysis of one selected miRNA, found over-expressed in ALS blood leucocytes (De Felice et al. 2014). Only for let7b-5p is there a partial overlapping with Freischmidt and colleagues' study, which reported a decrease of let7b in CSF from ALS patients, even though it did not reach statistical significance (Freischmidt et al. 2013).

Regarding other neurodegenerative diseases, two studies reported a significant downregulation in expression levels of both miR15b-5p and miR181a-5p in CSF from AD patients (Cogswell et al. 2008; Burgos et al. 2014). Thus, in CSF from ALS patients, the levels of miR15b-5p seem to follow the same trend described in AD patients, whereas the deregulation of miR181a-5p shows an opposite tendency.

Among the significantly deregulated miRNAs, miR181a-5p, miR15b-5p and miR21-5p showed the highest sensitivity and specificity in differentiating ALS from age-matched controls. Moreover, sensitivity and specificity of these miRNAs were increased when they were paired combining the upregulated miRNA with the downregulated miRNAs (miR181a-5p/miR15b-5p and miR181a-5p/miR21-5p). Thus, miR15b-5p, miR21-5p and miR181a-5p might be strong candidates for new ALS biomarkers. Furthermore, the observation that miR181a-5p was

downregulated in CSF from AD patients increased the application potential of this miRNA as specific marker of ALS.

In the present study, no associations were found between miRNA levels and ALS clinical variables. This result can be affected by the relatively limited number of patients included in each group. It is worth noting that in the case of survival analysis, the contemporary presence of low miR15b-5p and miR21-5p levels and high miR181a-5p levels was related to a shorter disease duration, even though it did not reach the statistical significance. Further analyses in a larger cohort of ALS patients will allow confirming or ruling out the possible association of these three deregulated miRNAs with the disease duration.

Moreover, no significant differences were present when miRNA levels of patients carrying the *C9orf72* expansion were compared with those of patients without the expansion. Although the analysis was performed in a relatively small number of patients, these data suggest a possible mutation-independent deregulation of CSF miRNAs. This is in line with the results obtained in a recent study of miRNA in the serum of sporadic ALS patients, in which deregulated miRNAs revealed the same trend in patients with or without mutations in the major ALS genes, including *C9orf72* expansion (Freischmidt et al. 2014). Although a subsequent study performed by the same research group reported a higher heterogeneity in serum miRNA pattern in sporadic ALS patients, the authors confirmed that more than 60 % of sporadic ALS samples shared a serum miRNA fingerprint with genetic cases (Freischmidt et al. 2015). It is hypothesized the presence of common pathogenic denominators, possibly related to a polygenetic background, conferring susceptibility for ALS and connecting defects in the majority of sporadic ALS and in ALS associated with mutation in different genes (Freischmidt et al. 2015).

The present study has some limitations and strengths. First, the approach used to identify deregulated miRNAs can affect the results. We have employed qRT-PCR which is a highly sensitive technique, but cannot identify novel miRNAs. Thus, it is possible that other informative miRNAs exist in CSF. On the other hand, validation experiments showed that this method is not only sensitive and specific

but also reproducible and accurate to detect miRNAs in CSF. In addition, all the steps of the qRT-PCR have been monitored by quality control procedures, to ensure the reliability of the results. A second challenge in miRNA quantification is data normalization that is a key point for an objective evaluation of expression levels. For miRNA analysis in CSF, no consensus internal controls have been established yet. We used two endogenous reference miRNAs (miRNA608 and miR328-3p), selected as the most stable and reproducible across different samples and experiments, and an exogenous control (Cel-miR-39-3p), widely reported in the literature. The analyses performed using the three reference miRNAs led to the same results. Regarding the individuals enrolled in the study, a strength is that all patients were clinically and genetically characterized and followed up and control subjects were matched to cases so as to eliminate the potential confounding effects of age and gender. A limitation is the relatively small number of patients enrolled in the study. The experiments reported here demonstrate that differentially expressed miRNAs in CSF represent informative markers able to distinguish ALS patients from unaffected control subjects; however, the results have to be confirmed in a larger cohort of patients. In addition, replication studies will allow to increase the number of ALS patients and to stratify them based on clinical and genetic features, to better evaluate the potential associations of miRNAs with these variables. Finally, in this study CSF miRNA levels of ALS patients have been compared to those of control subjects not affected by neurological disorders. It will be of fundamental importance to extend the comparison to patients affected by other neurodegenerative diseases, to evaluate the specificity of deregulated miRNAs as ALS biomarkers.

Besides their role as potential biomarkers, the deregulated miRNAs could reflect the biology of the tissue of origin, providing important insight into disease processes responsible for motor neuron degeneration. The majority of miRNAs analysed in this study are molecules with well-known functions, and the information about their potential role derives mainly from studies of brain tumours. Abundant data have recently shown a very tight connection between miR21 and miR15, and the use of these two miRNA in combination has been proposed as specific signature for glioma (Baraniskin et al. 2012).

Further studies reported a significant upregulation of miR21 expression levels in CSF from patients with glioblastoma (Akers et al. 2013, 2015), supporting the hypothesis that miR21 could be a marker of glial cell proliferation (Garg et al. 2015). Finally, miR181a-5p has been reported as downregulated in neuroblastoma, glioblastoma and glioma (Conti et al. 2009; Shi et al. 2008; Gibert et al. 2014). It is worth noting that in brain tumours, the deregulation of these three miRNAs displays a trend opposite of that observed in ALS in our study, where miR181a-5p is upregulated and miR21-5p and miR15b-5p are downregulated. It has been proposed that in the glioma, glioblastoma and astrocytoma carcinogenesis, these three miRNAs may act in combination as promoters of glial cell proliferation (Shi et al. 2008; Conti et al. 2009; Teplyuk et al. 2012). In particular, miR21-5p and miR-15b have been described as oncomirs, since they work as oncogenes and their overexpression leads to tumour growth, inhibiting the expression of tumour suppressor genes (Papagiannakopoulos et al. 2008; Pang et al. 2015). The miR181a-5p has been suggested as an anti-oncomir, which acts as a tumour suppressor in normal tissues, that triggers growth inhibition, apoptosis, and inhibits invasion (Conti et al. 2009). In the light of this evidence, it can be hypothesized that, whereas the contemporary downregulation of miR181a-5p and upregulation of miR21-5p and miR15b-5p are associated with apoptotic mechanism inhibition and cell proliferation, the contemporary upregulation of miR181a-5p and downregulation of miR21-5p and miR15b-5p we find could be linked to cell death by favouring apoptotic pathways. This deregulation could directly involve microglia, which in turn may contribute to neuronal damage. Functional in vitro studies will allow verifying though these deregulated miRNAs are related to apoptotic mechanisms responsible for cell death in ALS.

In summary, considering the high diagnostic value of combined miR181a-5p, miR15b-5p and miR21-5p analyses in this pilot study, we provide initial evidence that identified miRNAs could represent promising biomarkers for ALS. If replicated in a larger cohort of patients, these molecules may provide a valuable diagnostic tool. In perspective, these miRNAs may be used as prognostic biomarkers and as indicator of disease progression, to facilitate clinical management of this disease. Moreover, besides of the potential role of miRNAs as

biomarkers, the finding of a deregulated miRNA expression in patients with ALS may provide important insights about the pathogenesis of the disease and eventually contribute to develop potential future therapeutic approaches.

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Compliance with Ethical Standards

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References

Akers, J. C., Ramakrishnan, V., Kim, R., Phillips, S., Kaimal, V., Mao, Y., et al. (2015). miRNA contents of cerebrospinal fluid extracellular vesicles in glioblastoma patients. *Journal of Neuro-Oncology, 123*(2), 205–216.

Akers, J. C., Ramakrishnan, V., Kim, R., Skog, J., Nakano, I., Pingle, S., et al. (2013). MiR-21 in the extracellular vesicles (EVs) of cerebrospinal fluid (CSF): a platform for glioblastoma biomarker development. *PLoS ONE, 8*(10), e78115.

Andersen, P. M., & Al-Chalabi, A. (2011). Clinical genetics of amyotrophic lateral sclerosis: what do we really know? *Nature Reviews Neurology, 7*(11), 603–615.

Baraniskin, A., Kuhnhenn, J., Schlegel, U., Maghnouj, A., Zöllner, H., Schmiegel, W., et al. (2012). Identification of microRNAs in the cerebrospinal fluid as biomarker for the diagnosis of glioma. *Neuro-Oncology, 14*(1), 29–33.

Bartel, D. P. (2009). MicroRNAs: Target recognition and regulatory functions. *Cell, 136*(2), 215–233.

Brooks, B. R., Miller, R. G., Swash, M., & Munsat, T. L. (2000). World Federation of Neurology Research Group on Motor Neuron Diseases. El Escorial revisited: revised criteria for the diagnosis of amyotrophic lateral sclerosis. *Amyotroph Lateral Sclerosis and Other Motor Neuron Disorders, 1*(5), 293–299.

Burgos, K. L., Javaherian, A., Bomprezzi, R., Ghaffari, L., Rhodes, S., Courtright, A., et al. (2013). Identification of extracellular miRNA in human cerebrospinal fluid by next-generation sequencing. *RNA, 19*(5), 712–722.

Burgos, K., Malenica, I., Metpally, R., Courtright, A., Rakela, B., Beach, T., et al. (2014). Profiles of extracellular miRNA in cerebrospinal fluid and serum from patients with Alzheimer's and Parkinson's diseases correlate with disease status and features of pathology. *PLoS ONE, 9*(5), e94839.

Cogswell, J. P., Ward, J., Taylor, I. A., Waters, M., Shi, Y., et al. (2008). Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. *Journal of Alzheimer's Disease, 14*(1), 27–41.

Conti, A., Aguennouz, M., La Torre, D., Tomasello, C., Cardali, S., Angileri, F. F., et al. (2009). miR-21 and 221 upregulation and miR-181b downregulation in human grade II-IV astrocytic tumors. *Journal of Neuro-Oncology, 93*(3), 325–332.

De Felice, B., Annunziata, A., Fiorentino, G., Borra, M., Biffali, E., Coppola, C., et al. (2014). miR-338-3p is over-expressed in blood, CFS, serum and spinal cord from sporadic amyotrophic lateral sclerosis patients. *Neurogenetics, 15*(4), 243–253.

De Felice, B., Guida, M., Guida, M., Coppola, C., De Mieri, G., & Cotrufo, R. (2012). A miRNA signature in leukocytes from sporadic amyotrophic lateral sclerosis. *Gene, 508*(1), 35–40.

Denk, J., Boelmans, K., Siegismund, C., Lassner, D., Arlt, S., & Jahn, H. (2015). MicroRNA profiling of CSF reveals potential biomarkers to detect alzheimer's disease. *PLoSOne, 10*(5), e0126423.

Droppelmann, C. A., Campos-Melo, D., Ishtiaq, M., Volkening, K., & Strong, M. J. (2014). RNA metabolism in ALS: When normal processes become pathological. *Amyotroph Lateral Sclerosis and Other Motor Neuron Disorders, 15*(5–6), 321–336.

Eitan, C., & Hornstein, E. (2016) Vulnerability of microRNA biogenesis in FTD-ALS. *Brain Res pii: S0006-8993(16)00005-6. doi: 10.1016/j.brainres.2015.12.063*.
AQ2

Freischmidt, A., Müller, K., Ludolph, A. C., & Weishaupt, J. H. (2013). Systemic dysregulation of TDP-43 binding microRNAs in amyotrophic lateral sclerosis. *Acta Neuropathologica Communications, 1*, 42.

Freischmidt, A., Müller, K., Zondler, L., Weydt, P., Mayer, B., von Arnim, C. A., et al. (2015). Serum microRNAs in sporadic amyotrophic lateral sclerosis. *Neurobiology of Aging, 36*(9), 2660.e15-20.

Freischmidt, A., Müller, K., Zondler, L., Weydt, P., Volk, A. E., Božič, A. L., et al. (2014). Serum microRNAs in patients with genetic amyotrophic lateral sclerosis and pre-manifest mutation carriers. *Brain, 137*(Pt11), 2938–2950.

Gallego, J. A., Gordon, M. L., Claycomb, K., Bhatt, M., Lencz, T., & Malhotra, A. K. (2012). In vivo microRNA detection and quantitation in cerebrospinal fluid. *Journal of Molecular Neuroscience, 47*(2), 243–248.

Garg, N., Vijayakumar, T., Bakhshinyan, D., Venugopal, C., & Singh, S. K. (2015). MicroRNA regulation of brain tumour initiating cells in central nervous system tumours. *Stem Cells International*, 141793.

Gibert, B., Delloye-Bourgeois, C., Gattolliat, C. H., Meurette, O., Le Guernevel, S., Fombonne, J., et al. (2014). Regulation by miR181 family of the dependence receptor CDON tumor suppressive activity in neuroblastoma. *Journal of the National Cancer Institute, 106*(11), 318.

Goodall, E. F., Heath, P. R., Bandmann, O., Kirby, J., & Shaw, P. J. (2013). Neuronal dark matter: The emerging role of microRNAs in neurodegeneration. *Frontiers In Cellular Neuroscience, 7*, 178.

~~Ha, M., & Kim, V. N. (2014). Regulation of microRNA biogenesis. *Nature Reviews Molecular Cell Biology*, *15*(8), 509–524~~.

Haghikia, A., Haghikia, A., Hellwig, K., Baraniskin, A., Holzmann, A., Décard, B. F., et al. (2012). Regulated microRNAs in the CSF of patients with multiple sclerosis: A case-control study. *Neurology, 79* (22), 2166–2170.

~~Krichevsky, A. M., & Gabriely, G. (2009). miR-21: A small multi-faceted RNA. *Journal of Cellular and Molecular Medicine*, *13*(1), 39–53~~.

~~Li, L. C. (2008). The multifaceted small RNAs~~. ~~*RNA Biology,* 5(2), 61–64~~.

Maciotta, S., Meregalli, M., & Torrente, Y. (2013). The involvement of microRNAs in neurodegenerative diseases. *Front Cell Neurosci, 7*, 265.

Marangi, G., & Traynor, B. J. (2015). Genetic causes of amyotrophic lateral sclerosis: New genetic analysis methodologies entailing new opportunities and challenges. *Brain Research, 1607*, 75–93.

Millecamps, S., Boillée, S., Le Ber, I., Seilhean, D., Teyssou, E., Giraudeau, M., et al. (2012). Phenotype difference between ALS patients with expanded repeats in C9ORF72 and patients with mutations in other ALS-related genes. *Journal of Medical Genetics, 49*(4), 258–263.

Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., et al. (2008). Circulating microRNAs as stable blood-based markers for cancer detection. *Proceedings of the National Academy of Sciences of the United States of America, 105*(30), 10513–10518.

Müller, M., Kuiperij, H. B., Claassen, J. A., Küsters, B., & Verbeek, M. M. (2014). MicroRNAs in Alzheimer's disease: Differential expression in hippocampus and cell-free cerebrospinal fluid. *Neurobiology of Aging, 35*(1), 152–158.

Pacifici, M., Delbue, S., Kadri, F., & Peruzzi, F. (2014). Cerebrospinal fluid MicroRNA profiling using quantitative real time PCR. *Journal of Visualized Experiments, 83*, e51172.

Paez-Colasante, X., Figueroa-Romero, C., Sakowski, S. A., Goutman, S. A., & Feldman, E. L. (2015). Amyotrophic lateral sclerosis: mechanisms and therapeutics in the epigenomic era. *Nature Reviews Neurology, 11*(5), 266–279.

Pang, C., Guan, Y., Zhao, K., Chen, L., Bao, Y., Cui, R., et al. (2015). Up-regulation of microRNA-15b correlates with unfavorable prognosis and malignant progression of human glioma. *International Journal of Clinical and Experimental Pathology, 8*(5), 4943–4952.

Papagiannakopoulos, T., Shapiro, A., & Kosik, K. S. (2008). MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells. *Cancer Research, 68*(19), 8164–8172.

Pritchard, C. C., Cheng, H. H., & Tewari, M. (2012). MicroRNA profiling: Approaches and considerations. *Nature Reviews Genetics, 13*(5), 358–369.

Rao, P., Benito, E., & Fischer, A. (2013). MicroRNAs as biomarkers for CNS disease. *Frontiers in Molecular Neuroscience, 6*, 39.

Renton, A. E., Chiò, A., & Traynor, B. J. (2014). State of play in amyotrophic lateral sclerosis genetics. *Nature Neuroscience, 17*(1), 17–23.

Robberecht, W., & Philips, T. (2013). The changing scene of amyotrophic lateral sclerosis. *Nature Reviews Neuroscience, 14*(4), 248–264.

Roberts, R. A., Aschner, M., Calligaro, D., Guilarte, T. R., Hanig, J. P., Herr, D. W., et al. (2015). Translational biomarkers of neurotoxicity: A health and environmental sciences institute perspective on the way forward. *Toxicological Sciences, 148*(2), 332–340.

Sheinerman, K. S., Tsivinsky, V. G., Crawford, F., Mullan, M. J., Abdullah, L., & Umansky, S. R. (2012). Plasma microRNA biomarkers for detection of mild cognitive impairment. *Aging, 4*(9), 590–605.

Shi, L., Cheng, Z., Zhang, J., Li, R., Zhao, P., Fu, Z., et al. (2008). hsa-mir-181a and hsa-mir-181b function as tumor suppressors in human glioma cells. *Brain Research, 1236*, 185–193.

~~Smith, B. N., Newhouse, S., Shatunov, A., Vance, C., Topp, S., Johnson, L., et al. (2013)~~. ~~The C9ORF72 expansion mutation is a common cause of ALS ± FTD in Europe and has a single founder. *European Journal of Human Genetics, 21*(1), 102–108~~.

Teplyuk, N. M., Mollenhauer, B., Gabriely, G., Giese, A., Kim, E., Smolsky, M., et al. (2012). MicroRNAs in cerebrospinal fluid identify glioblastoma and metastatic brain cancers and reflect disease activity. *Neuro-Oncology, 14*(6), 689–700.

Teunissen, C. E., Tumani, H., Engelborghs, S., & Mollenhauer, B. (2014). Biobanking of CSF: International standardization to optimize biomarker development. *Clinical Biochemistry, 47*(4–5), 288–292.

Wahid, F., Shehzad, A., Khan, T., & Kim, Y. Y. (2010). MicroRNAs: synthesis, mechanism, function, and recent clinical trials. *Biochimica et Biophysica Acta, 1803*(11), 1231–1243.

Xie, F., Xiao, P., Chen, D., Xu, L., & Zhang, B. (2012). miRDeepFinder: a miRNA analysis tool for deep sequencing of plant small RNAs. *Plant Molecular Biology* (Epub ahead of print).

AQ1