Circulating miR-181a-5p is a prognostic biomarker for amyotrophic lateral sclerosis

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One Sentence Summary: higher plasma miR-181a-5p levels increase mortality risk in ALS patients.

Abstract (197 words)

Amyotrophic lateral sclerosis (ALS) is a relentless neurodegenerative disease affecting the motor neuron system. Variability in the rate of disease progression has limited the effectiveness of ALS clinical trials. Thus, better outcome measures are desperately needed in order to achieve therapeutic progress. Here, we investigate the potential of plasma cell-free microRNAs as biomarkers to predict ALS progression. We apply an unbiased high-throughput approach to define miRNA levels in a large cohort of ALS patients. Crucially, we conduct our analysis also on longitudinal samples reflecting disease progression, and are able to integrate detailed clinical phenotyping in our analysis. We find miR-181a-5p levels to be stable throughout the disease course and demonstrate that high miR-181a-5p plasma levels predict shortened survival in ALS patients, with a 2.5 fold reduction in median survival for patients with high miR-181a-5p plasma levels. We replicated this finding in an independent validation cohort, where an eight-fold (×8) difference in miR-181a-5p levels between the two prognosis subgroups was robustly measurable by quantitative real time PCR. In conclusion, miR-181a-5p plasma levels can predict disease course in ALS, identifying it as a novel biomarker for patient stratification with the potential to greatly enhance the effectiveness of clinical trials.

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder of the motor neuron system, for which no effective disease-modifying treatment exists. ALS is characterized by a significant variability in progression rates [1,2], which is a significant challenge for patient stratification in clinical trials. Reliable biomarkers of disease progression would therefore be invaluable for ALS patient stratification prior to enrolment in clinical trial. Ideal biomarkers should remain stable during the course of disease, be detectable in accessible tissue, and also be easily measurable. To date, intensive research has identified only a few potential blood-based ALS biomarkers [3-5], including cell-free neurofilaments [6-8], and pro-inflammatory cytokines [9-11]. microRNAs (miRNAs) are endogenous non-coding RNAs that are essential for motor neuron survival and have been shown to be globally downregulated in post mortem ALS motor neurons [12-14]. While circulating miRNA profiles have been previously characterized in ALS [15-17], the potential of miRNA biomarkers for ALS prognosis, and as readouts of disease progression has not yet been investigated. Here, we take an hypothesis-free approach by applying next generation sequencing (NGS) to comprehensively study plasma miRNAs in a large cohort of ALS cases. We identified one miRNA, miR-181a-5p, as being stable over time in individual patients. miR-181a-5p levels predict disease progression in an initial and second, validation cohort, demonstrating its use as a novel ALS biomarker.

Results

We used unbiased next generation sequencing to investigate, without an *a priori* bias, the comprehensive landscape of plasma miRNAs in ALS patients with available clinical information (Table 1).

In our search for a prognostic biomarker that is stable throughout the disease course, we experimentally assessed miRNA measurement variability (noise) over time, by analyzing a longitudinal sample cohort of 22 patients, with four longitudinal blood samples taken: (t_1-t_4) during the course of 30 months (2.5 years). 88 samples were prepared from total plasma RNA, as previously described [18], and profiled by small RNA next generation sequencing (RNA-seq). Linear miRNA quantification was achieved via unique 12-nucleotide molecular identifiers (UMIs). miRNAs with ≥50 UMIs in at least 60 percent (N=53) of the samples were considered above noise level. Thus, of 2008 miRNAs aligned to the human genome (GRCh37/hg19), 179 passed the threshold we set (see table S1). Measured variability between 22 individuals was low for 126/179 miRNAs (-log2 of t_4/t_1 standard error ≥ -0.2), and these were considered stable, while 53/179 other miRNAs exhibited higher variability between patients and were excluded from further analysis (Figure 1A, *y-axis*). For example, miR-181a-5p levels exhibited reduced variability at each time point across individual patients, in comparison with miR-1-3p (Fig. 1B, C).

miRNAs could be further characterized by intra-personal changes over time. Intriguingly, four miRNAs (miR-423/484/92a/b) exhibited a robust increase in abundance from t_1 to t_4 (Figure 1A, *x-axis*; Z-score of log2 t_4/t_1 > 1.5 SD), while four other miRNAs (miR-29/146/148/191) exhibited a decrease (Z-score of log2 t_4/t_1 < -1.5 SD). miR-423/484/92a/b modestly increased

early on (~1.15 fold t_2 or t_3 relative to t_1), and further increased ~1.7-fold upon the fourth sampling (t_4 / t_1), while miR-29/146/148/191 decreased by up to 1.6-fold upon the fourth sampling (Figure S1A-C). The correlation of miR-423/484/92a/b levels to disease progression was also observed when time from enrolment (in months) was considered as a continuous variable (Figure S2A-D), while those of miR-29/146/148/191 showed a non-significant correlation (Spearman $|\rho| < 0.2$, $p>0.05$; not shown) and were not followed up further. Individual trajectories of miR-423/484/92a/b exhibited increases in most of the patients from t_1 to t_4 (Figure S3E-H). Furthermore, these miRNAs increased significantly in a replication longitudinal cohort (N=22-24 patients, one-tailed t-test: miR-484, p=0.01; miR-92a-3p, p=0.006; miR-92b-3p, p=0.04; Figure S2J-L), whereas miR-423-5p did not replicate the upward trend in the second longitudinal cohort (one-tailed p-value=0.16, Figure S2I).

The relative D50 (rD50), is a modelled derivative of ALSFRS-R decay, that reveals the disease covered by individual patients independent of the rate of progression [19]. For example, an rD50 of 0.0 and 0.5 signify ALS onset and the time-point of halved functionality, respectively [19]. miR-484/92a/b levels measured at longitudinal blood samplings from the same patients correlated with rD50 at the time of sampling (Figure S3A-D) and may be considered as candidate molecular biomarkers which increase in all patients independent of individual disease aggressiveness.

We next explored potential blood-borne miRNAs for prognostic biomarkers. An ideal prognostic biomarker should be preferentially stable throughout the course of disease. We identified 118 miRNA candidate biomarkers, whose plasma levels were relatively stable during disease progression (green features in Figure 1A and table S1). We then correlated miRNA

expression at enrolment to survival duration, calculated as lifespan from either symptom onset or from first phlebotomy at study enrolment (on average 30.0±6 months after symptom onset). The levels of one miRNA, miR-181a-5p exhibited significant correlation to survival length (Spearman correlation coefficient = - 0.21, p=0.02, Figure 2A, S4A). Because miR-181a-5p levels remain stable in time (Figure 1A, B), it is unlikely that measurements are confounded by sampling at different disease stages. The stability of miR-181a-5p during disease progression is also indirectly implied by comparable miR-181a-5p levels between patients sampled at different (early stable, early progressive and late progressive) disease phases ($0 \leq rD50 < 0.25$; $0.25 \leq rD50 < 0.5$; $rD50 \geq 0.5$, respectively; ANOVA p=0.35, Figure S5A) and by lack of correlation between miR-181a-5p levels and rD50 at enrolment (Fig. S5B).

We further subdivided patients in quartiles according to the expression level of individual miRNA genes, and investigated how the low quartile (30 patients) compared to the rest of the cases (85 patients). Differences in plasma miR-181a-5p levels were able to predict survival length better than any other miRNA. Kaplan Meier curves revealed clear separation of survival between the subgroups, based on plasma miR-181a-5p levels at enrolment (log-rank test chi-square=8.6, p=0.003, Figure 2B and see also table S2 for number of surviving patients at each time point). The median patient survival in the lower miR-181a-5p quartile was 36 months, compared to 13 months at the higher three quartiles. Thus, plasma miR-181a levels predict a substantial median survival difference of 23 months that is equivalent to a 280% increase in survival length for patients with lower plasma miR-181a-5p levels. Comparable results were gained, when plasma miR-181a-5p levels at enrolment were stratified against survival from reported first symptoms (disease onset, log-rank chi-square=9.4, p=0.002; Figure 2C and table S2).

It is noteworthy that patient individual disease covered at enrolment (rD50) was comparable between the low quartile of miR-181a-5p and the rest of the cases (Figure S5C). Furthermore, enrolment progression rate, enrolment ALSFRS-R scores, and chronological age at onset were not correlated with miR-181a-5p levels, and these phenotypic features did not differ between the low and high miR-181a-5p quantiles, except for a significant but modest increase in enrolment progression rate (Figure S6A-F). These findings exclude the possibility that survival differences between miR-181a-5p expression bins result from factors other than miR-181a-5p expression levels, such as the clinical status of the patients when they are sampled.

The monthly mortality hazard ratio (HR) was calculated for different plasma miR-181a-5p quantiles and clinical covariates, by multivariate Cox regression analysis. This analysis allows calculation of an independent hazard ratio for each covariate while holding the other covariates constant. High miR-181a-5p levels (top three quartiles) exhibited a hazard ratio of 2.35 (95% CI 1.4-3.9), relative to the lowest quartile when calculated from enrolment (Wald test $p<0.001$, Figure 3A), and a hazard ratio of 2.4 when calculated from disease onset (95% CI, 1.5-4.0, $p<0.01$, Figure 3B). Enrolment progression rate, i.e. the rate of change in ALSFRS from symptom onset to enrolment, was also associated with a high hazard ratio of 2.16 for survival time from enrolment (95% CI 1.6-2.9, $p<0.001$, Figure 3A), as previously reported [20]. Taken together, higher miR-181a plasma levels at enrolment associate with a considerably poor prognosis in ALS, an effect which is not confounded by other phenotypic properties.

In order to validate our findings, we performed a replication study by measuring miR-181a-5p levels in plasma of an independent cohort of 56 patients (Table 1) by an orthogonal qPCR methodology. The replication study confirmed the existence of a prognostic gap of 180%, from 14 to 25 months, between patients with high or low plasma miR-181a-5p levels,

respectively (Log rank test: chi square=3.45, one-tailed p-value=0.03, Figure 4A and table S2). The survival difference was reproduced in the replication cohort, even when calculated from first reported symptoms (230%, 31 months from disease onset to 71 months, chi square=3.0, one-tailed p-value=0.04, Figure 4B and table S2). Importantly, the average miR-181a-5p levels varied by 8-fold between the plasma of patients with better prognosis, relative to those with poor prognosis (Figure 4C), suggesting a good dynamic range for biomarker quantification. Our data from two different cohorts of ALS patients therefore establish miR-181a-5p as a new cell-free prognostic biomarker for ALS.

In summary, we report the results of the most elaborated small RNA-seq study in ALS undertaken to date. We show that miRNAs appear to be mostly unchanged longitudinally during disease, with the exception of miR-423/484/92a/b, that increase over 30 months of disease. This feature could be used to monitor disease progression / individual disease covered and warrants further exploration as pharmacodynamic biomarkers of response to therapy. Crucially, we find that miR-181a-5p levels predict shortened survival in two separate ALS cohorts. Intriguingly, the median survival of patients with low miR-181a-5p plasma levels was more than doubled compared to patients with high miR-181a-5p. An eight-fold difference in miR-181a-5p levels between the two prognosis subgroups, is robustly measurable. Although the tissue source of miR-181a-5p is unknown, it emerges as a prognostic ALS biomarker that is able to improve patient stratification in clinical trials.

Online Methods

Standard protocol approvals, registrations, and patient consents

All participants provided written consent (or gave verbal permission for a carer to sign on their behalf) to be enrolled in the ALS biomarkers study and ethical approval was obtained from the East London and the City Research Ethics Committee 1 (09/H0703/27).

Study design

For survival analysis, 120 ALS patients were needed to obtain a hazard ratio of 3-fold in short survivors compared with long survivors, with a power of 99% and a p-value of 0.01. We determined the sample size based on these calculations. Because sample processing was done in different batches, samples were randomly allocated to the batches except for longitudinal ALS samples from the same individual, that were sequenced in the same batch to avoid batch-induced biases in interpretation of longitudinal changes. RNA-seq data from plasma at study enrolment were collected retrospectively, while phenotypic data on de-identified patients was separated and blinded.

Participants and sampling

This study included a first cohort with 115 patients with ALS from the ALS biomarker study. ALS patients were diagnosed according to standard criteria by experienced ALS neurologists [21], and were enrolled if they met inclusion criteria until the desired sample size was reached (consecutive series). Informed consent was obtained from all participants. Serial plasma samples and clinical information were obtained, on average, every 2 to 4 months from 22 of the 115 patients with ALS. No selection criteria were applied to individuals with ALS sampled longitudinally, other than their willingness to donate further samples. Symptom onset was

defined as first patient-reported weakness. Disease severity was assessed with the revised ALS Functional Rating Scale (ALSFRS-R)[22], and progression rate at enrolment (i.e. first blood draw) was calculated as follows: (48 - enrolment ALSFRS-R)/time (in months) from symptom onset to enrolment. Progression was also modeled using the D50 model which fits a sigmoid decay across all available ALSFRS-R scores [19,23]. Use of Riluzole (or not) at the time of sampling was recorded. In samples from cohort I (115 ALS patients), miRNAs were profiled with NGS. A second cohort of 56 ALS patients, was used to validate the results of the first cohort by an orthogonal method of quantitative real time PCR. Plasma samples were stored in -80^{0}C until RNA extraction and subsequent small RNA next generation sequencing.

Small RNA next generation sequencing

Total RNA was extracted from plasma using the miRNeasy micro kit (Qiagen, Hilden, Germany) and quantified with Qubit fluorometer using RNA broad range (BR) assay kit (Thermo Fisher Scientific, Waltham, MA). For small RNA next generation sequencing (NGS), libraries were prepared from 7.5 ng of total RNA using the QIAseq™ miRNA Library Kit and QIAseq miRNA NGS 48 Index IL (Qiagen), by an experimenter who was blinded to the identity of samples. Following 3' and 5' adapter ligation, small RNA was reverse transcribed, using unique molecular identifier (UMI), primers of random 12-nucleotide sequences. This way, precise linear quantification miRNA is achieved, overcoming potential PCR-induced biases [18]. cDNA libraries were amplified by PCR for 22 cycles, with a 3' primer that includes a 6-nucleotide unique index. Following size selection and cleaning of libraries with magnetic beads, quality control was performed by measuring library concentration with Qubit fluorometer using dsDNA high sensitivity (HS) assay kit (Thermo Fisher Scientific, Waltham, MA) and confirming library size

with Tapestation D1000 (Agilent). Libraries with different indices were multiplexed and sequenced on a single NextSeq 500/550 v2 flow cell (Illumina), with 75bp single read and 6bp index read. Fastq files were demultiplexed using the User-friendly Transcriptome Analysis Pipeline (UTAP) developed at the Weizmann Institute [24]. Human exact mature miRNA sequence, as defined by miRBase [25], were mapped using Geneglobe (Qiagen).

Quantitative real time PCR (qPCR)

TaqMan Advanced MicroRNA cDNA Synthesis Kit (Applied Biosystems) was used for cDNA reverse transcription (10 ng input). Universal PCR Master Mix without AmpErase UNG on white reaction plates (MicroAmp EnduraPlate Optical 96-well, Thermo Fisher) and run on a StepOnePlus machine (Applied Biosystems). TaqMan qPCR was performed using Advanced MicroRNA Assays (reaction volume 10 μL) with the following probes: hsa-miR-181a-5p (Assay ID: 477857_mir); hsa-miR-423-5p (Assay ID: 478090_mir); hsa-miR-484 (Assay ID: 478308_mir); hsa-miR-92a-3p (Assay ID: 477827_mir); hsa-miR-92b-3p (Assay ID: 477823_mir); hsa-miR-140-3p (Assay ID: 477908_mir) and hsa-miR-185-5p (Assay ID: 477939_mir) were selected as normalizers based on stable expression in the first cohort: (1) basemean expression between 500-3,000; (2) coefficient of variation ≤ 0.35 [26] and (3) minimal, insignificant, changes in patient survival between different expression bins of the miRNA. Relative miR-181a-5p quantity, normalized to the average expression of hsa-miR-140-3p and hsa-miR-185-5p were binned to lowest 25% and highest 75%, for Kaplan-Meier survival analysis. Relative miR-423/484/92a/92b quantities, normalized to same normalizers was compared between enrolment sample (t_1) and corresponding follow-up sample (t_2) by one-tailed paired t-test.

Statistical analysis

Plasma samples with ≥ 50,000 miRNA UMIs at enrolment (t_1) were included in the analysis of prognostic biomarkers, i.e. stratification of survival lengths according to low/high expression bins of miRNAs. NGS data was analyzed via DESeq2 package in R Project for Statistical Computing [27,28], under the assumption that miRNA counts followed negative binomial distribution and data were corrected for library preparation batch in order to reduce its potential bias. For longitudinal multiple time points analysis, correction was performed per subject. *P* values were calculated by Wald test [28,29] and adjusted for multiple testing according to Benjamini and Hochberg [30]. Log rank Mantel-Cox test was used for Kaplan-Meier survival estimators and a fixed date was used to censor data for survival analysis. Cox regression was used to calculate mortality hazard ratio, with miR-181a-5p levels and phenotypic features covariates. For longitudinal miRNA expression by qPCR, one-tailed paired t-test was used. Outliers were detected by Grubbs test [31] and excluded from analysis. Graphs were generated with GraphPad Prism 5.

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Competing interests

The authors state that they have no competing interests.

Data availability

Source data for figures 1,2 and 4 are provided with the paper.

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Magen et al Table 1

	Cohort I	Cohort II
Number of patients (% males)	115 (64.3%)	56 (48.2%)
Age at enrolment	64±1 yr.	65±2 yr.
Age of onset (1st reported symptoms)	61±1 yr.	62±2 yr.
Disease duration at enrolment	30±3 m.	35±6 m.
ALSFRS-R at enrolment	36±1	37±1
El-Escorial (Definite/probable/lab-supported/possible)	34/51/15/15	3/14/21/15/3
Bulbar onset / total	38/115	17/56
Riluzole treated / total	80/115	34/56
ALS Family history / total	7/115	unknown
C9ORF72 genetics / total	8/115	2/56

Table 1. Summary of demographic and clinical characteristics of ALS Cohorts I and II samples. ALSFRS-R: ALS functional rating scale.

Magen et al Figure 1

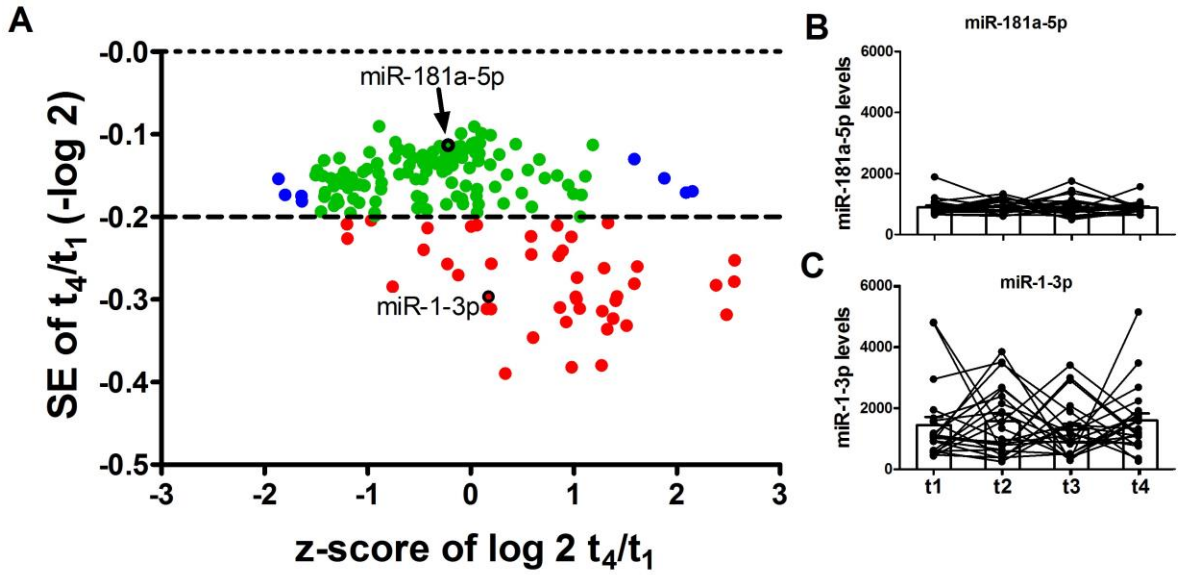

Figure 1. Assessment of miRNAs signal and noise in longitudinal samples from Patients with ALS. (A) The x-axis denotes the normalized change in miRNA levels between first and last measurements (as log 2-transformed t_4/t_1 ratios), relative to the average change of all 179 sequenced miRNAs. The y-axis denotes the variability in measurements, per-miRNA for 179 sequenced species between the 22 individuals (- log 2-transformed values of the standard error of t_4/t_1 ratios). Green, red features: are above or below the threshold of stability, respectively (-0.2). Blue features: stable miRNAs that are increasing progressively with disease in individual patients > 1.5 SD (miR-423/484/92a/b) or < -1.5 SD (miR-29/146/148/191) from the average miRNA. **(B-C)** Spaghetti plots of individual trajectories over four longitudinal sample collections for **(B)** miR-181a-5p and **(C)** miR-1-3p.

Magen et al Figure 2

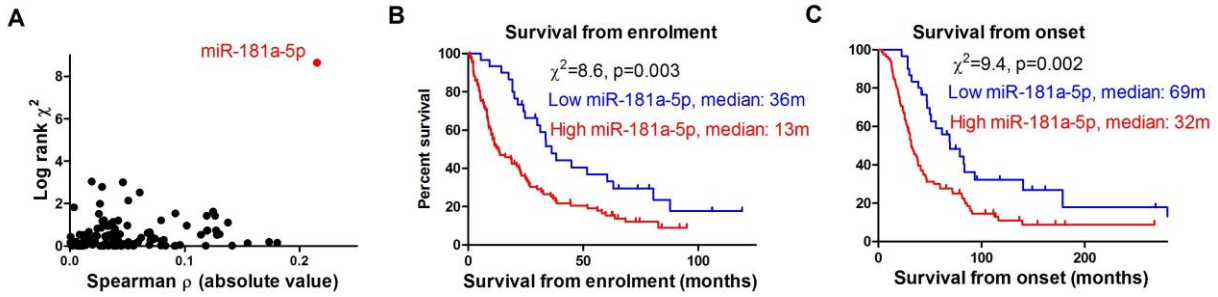

Figure 2. Prognostic value of miR-181a-5p in predicting ALS patient survival. (A) Survival predictive value of individual miRNA levels in plasma, binned by lower quantile vs. higher 3 quantiles (logrank test, *y-axis*), for miRNAs depicted in Figure 1 as 'stable'. Data plotted against miRNA level correlation to survival length (absolute value of Spearman correlation-coefficient, *x-axis*). Cumulative survival (Kaplan-Meier) curves from **(B)** enrolment, or from **(C)** reported first symptoms for patients, subdivided by miR-181a-5p levels, at low quartile (30 patients) compared to the rest of the cases (85 patients).

Magen et al Figure 3

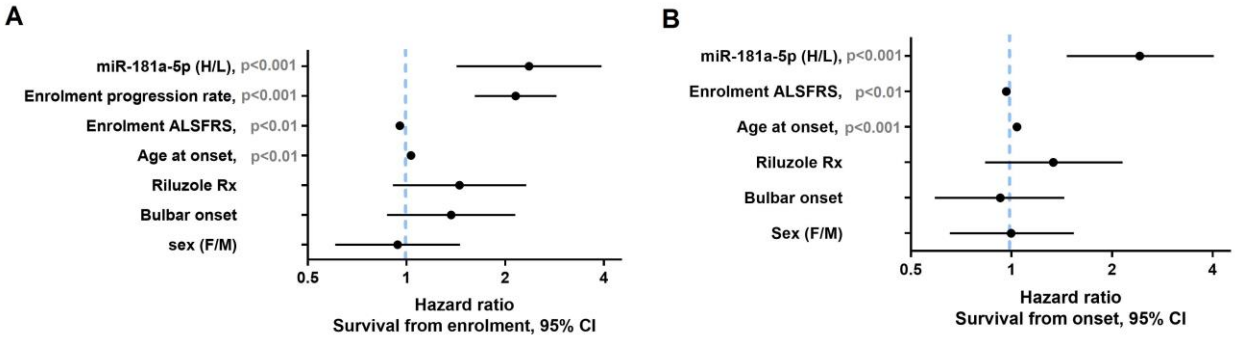

Figure 3. Mortality hazard ratio for miR-181a-5p. Mortality hazard ratio calculated by survival length from enrolment **(A)**, or from disease onset **(B).** Cox-regression analyses of covariates: miR-181a-5p levels, enrolment progression rate, enrolment ALSFRS-R score, age at onset, Riluzole-treatment, Bulbar onset, sex (Female/Male). Light blue dashed-line denotes unchanged mortality. Mean ± 95% CIs.

Magen et al Figure 4

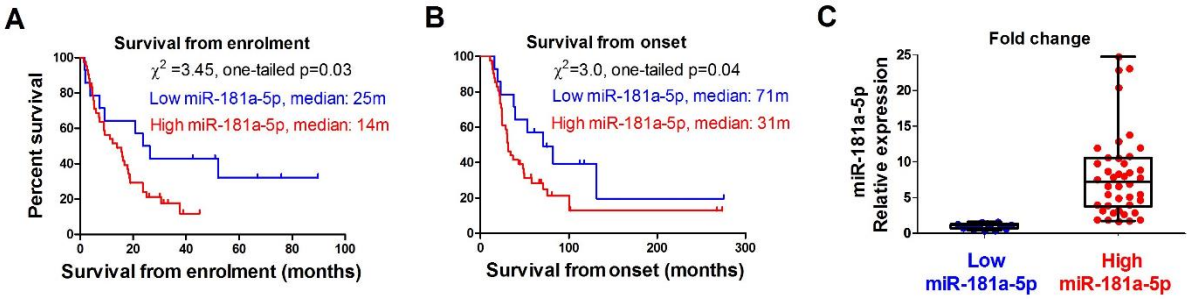

Figure 4. Prognostic value of miR-181a-5p is replicated in an independent cohort and by an orthogonal measuring technique. Kaplan-Meier curves from **(A)** enrolment, or **(B)** from reported first symptoms for a replication cohort, subdivided by miR-181a-5p levels, at low (14 patients) vs. high (42 patients) quantiles. (**C**) Scatter plot of plasma miR-181a-5p levels at low vs. high quantiles.

Supplementary figures and tables

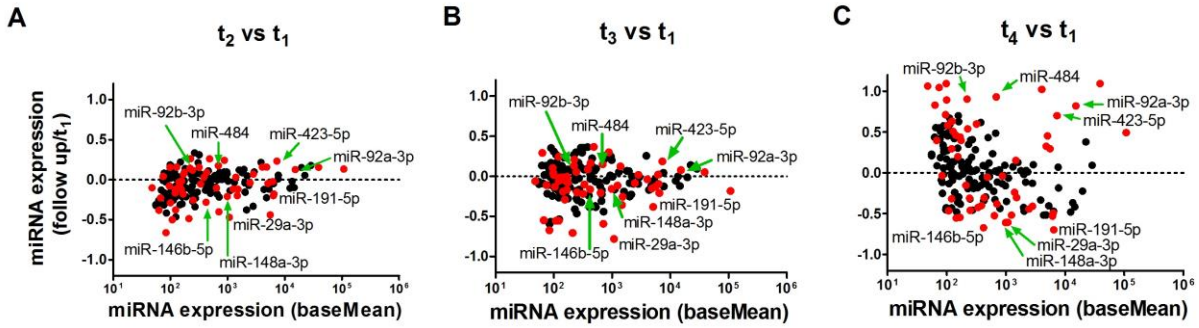

Figure S1. (A-C) MA plots of differential miRNA expression upon repeated sampling relative to the first phlebotomy. Red features denote miRNAs with statistically significant change in levels.

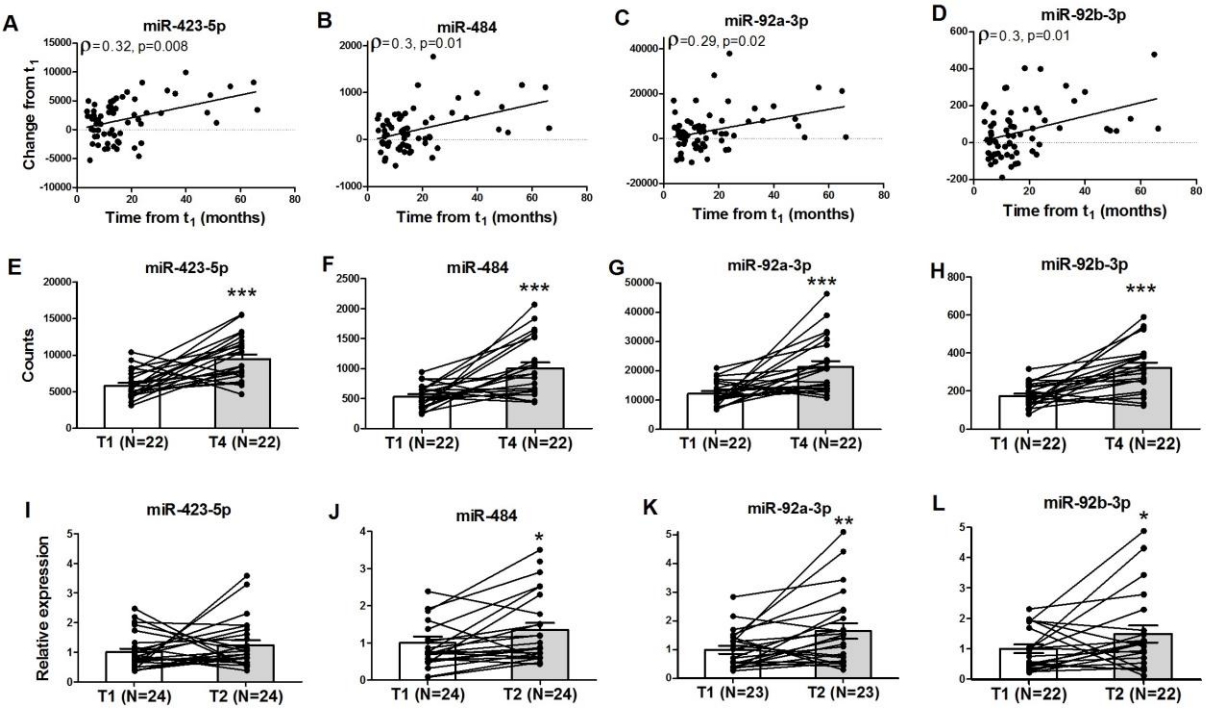

Figure S2. Longitudinal changes in miRNAs in ALS. Temporal changes in the levels of **(A)** miR-423-5p, **(B)** miR-484, **(C)** miR-92a-3p, or **(D)** miR-92b-3p and **(E-H)** corresponding spaghetti plots of individual trajectories (t_1-t_4). Time intervals: t_1-t_2 6.3±0.3 m.; t_1-t_3 13.0±0.3 m.; t_1=t_4 32.7±3 m. Disease duration: t_1 28.8±3 m.; t_4 61.5±3 m. Replication study of **(I)** miR-423-5p **(J)** miR-484 **(K)** miR-92a-3p or **(L)** miR-92b-3p levels by qPCR at second/first phlebotomy (t_1-t_2) in individual patients. Mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, one-tailed paired t-test or Wald test.

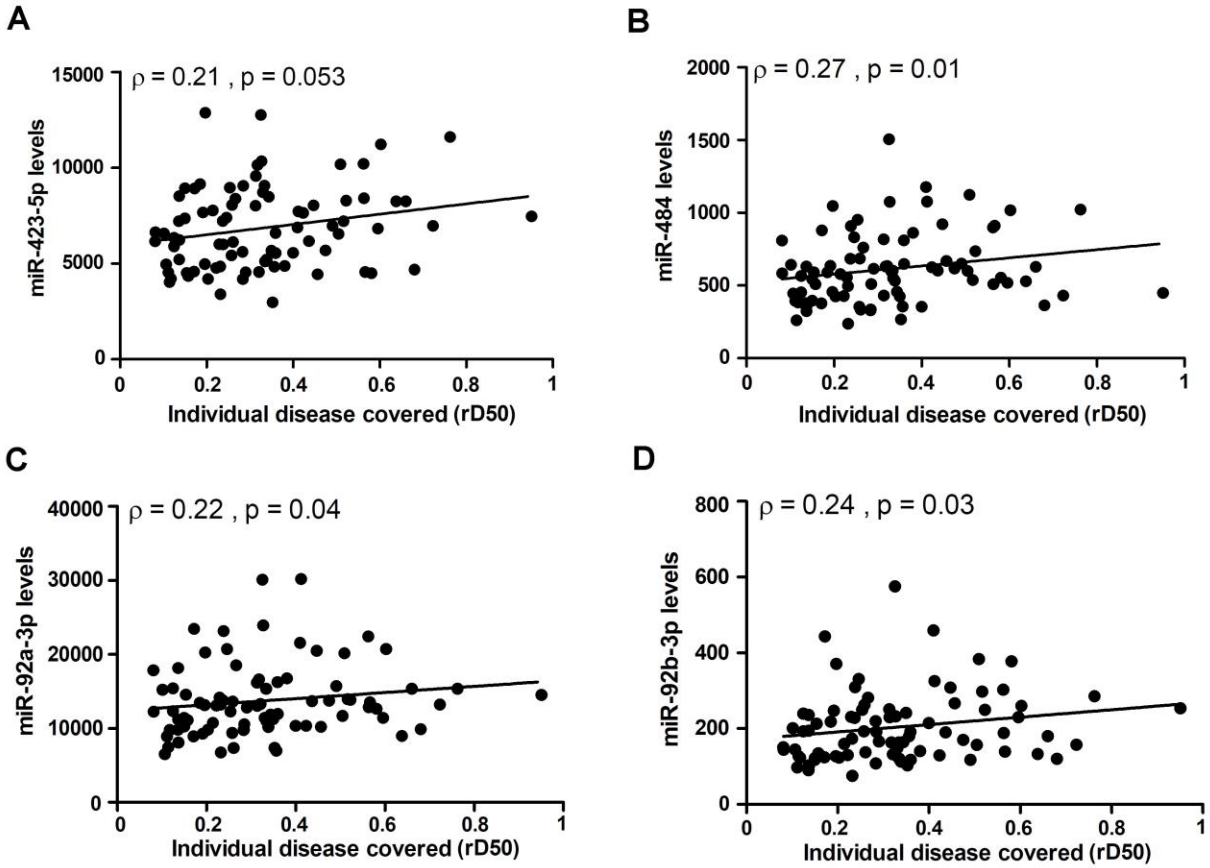

Figure S3. Longitudinally changed miRNAs increase with disease progression. Correlation between the relative disease covered (rD50) in longitudinal plasma collections (X-axis) and levels of **(A)** miR-423-5p, **(B)** miR-484, **(C)** miR-92a-3p, and **(D)** miR-92b-3p.

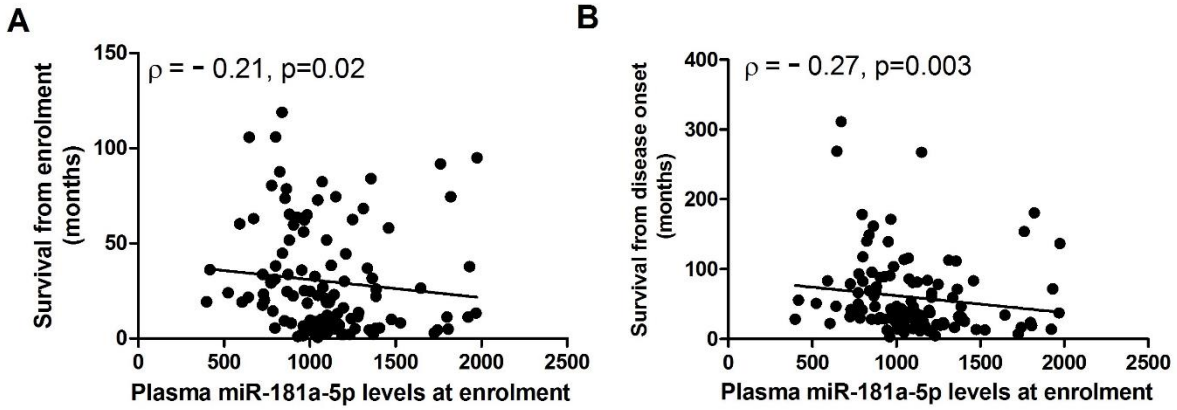

Figure S4. Plots depicting inverse correlation between miR-181a-5p and survival from first phlebotomy (A), or from disease onset (B). When the three samples taken from subjects whose survival from onset was above 260 months, Spearman correlation slightly decreases (from -0.27 to -0.25) and the p-values marginally increases (from 0.003 to 0.008), suggesting the overall data and interpretation remain unchanged.

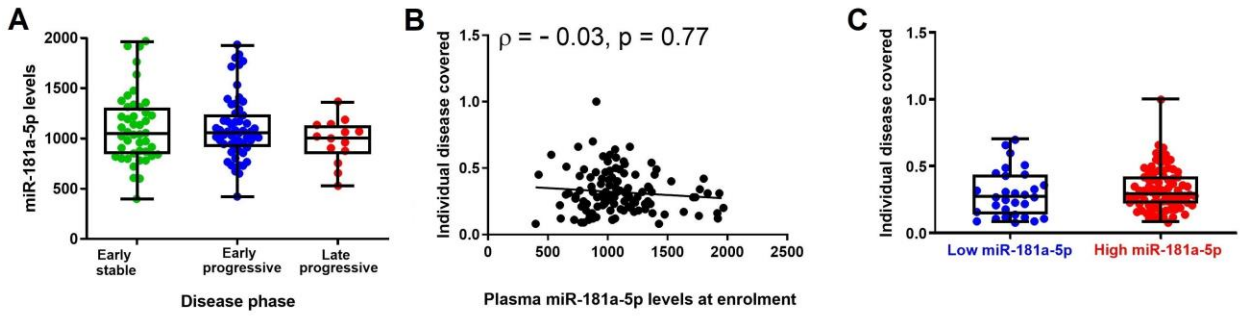

Figure S5. miR-181a-5p levels are not associated with disease phase. **(A)** miR-181a-5p in different phases of disease defined by rD50 values. One-way ANOVA: F (2,112) = 1.06, p=0.35. **(B)** correlation of miR-181a-5p levels with rD50 **(C)** rD50 differences between low and high miR-181a-5p expression bins.

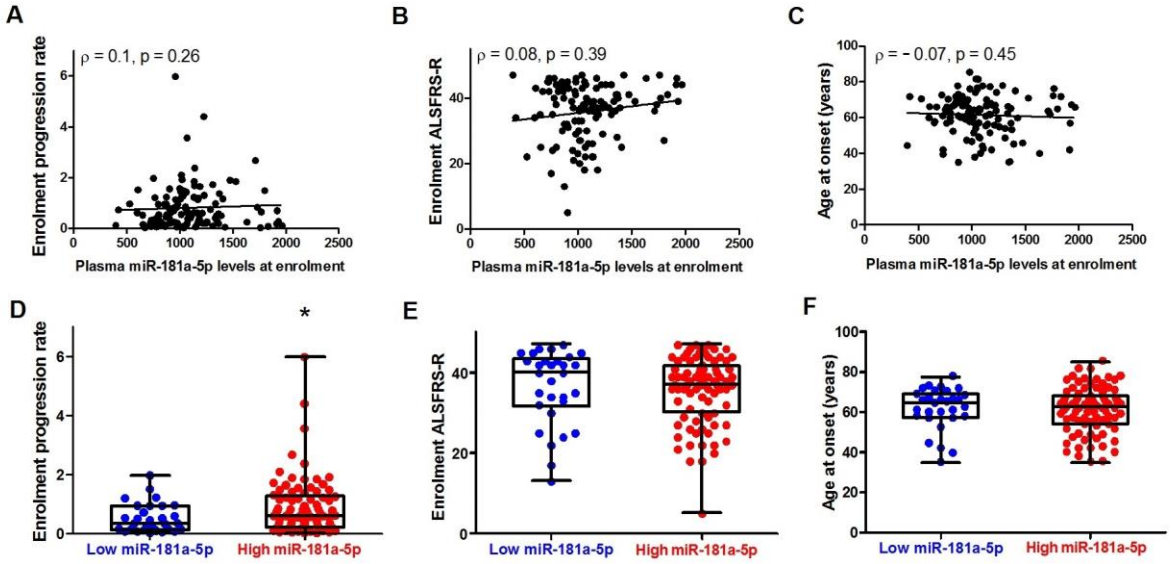

Figure S6. Correlation of miR-181a-5p levels at enrolment with phenotypic properties, including **(A))** progression rate at enrolment **(B)** enrolment ALSFRS-score, and **(C)** patient age at disease onset. Differences in these properties between low and high miR-181a bins **(D-F)**. *p<0.05 high vs low, Mann-Whitney U test.