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The role of hsa-miR-21-5p, hsa-miR-210-3p, hsa-miR-197-3p, hsa-miR-125a-5p and hsa-miR-206-3p as circulating microRNA signatures in amyotrophic lateral sclerosis

Maria Sokratous
University of Thessaly
Polyxeni Stamati
University of Thessaly
Ioannis Liampas
University of Thessaly
Chrysoula Marogianni
University of Thessaly
Panagiotis Liakos
University of Thessaly
Dimitrios Bogdanos
University of Thessaly
Efthimios Dardiotis
University of Thessaly
Vasileios Siokas
vsiokas@med.uth.gr
University of Thessaly

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Abstract

Background

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder distinguished by selective loss of motor neurons. Up to now, robust biomarkers for diagnosis and disease progression are missing. This study aims to examine the expression of hsa-miR-21-5p, hsa-miR-210-3p, hsa-miR-197-3p, hsa-miR-125a-5p and hsa-miR-206-3p, as candidate ALS biomarkers in the cerebrospinal fluid (CSF) and serum.

Methods

CSF and serum samples were collected from 50 ALS patients, 50 age- and sex-matched healthy controls and 50 disease-controls. The expression of the selected miRNAs was quantified by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Associations with clinical parameters, including disease phenotype, amyotrophic lateral sclerosis functional rating scale - revised (ALSFRS-R) at diagnosis, disease duration and progression rate, were evaluated. Functional enrichment analysis was performed on predicted miRNA target genes using Gene Set Enrichment Analysis (GSEA) with Reactome, Gene Ontology Biological Process and Hallmark gene sets.

Results

Hsa-miR-210-3p, hsa-miR-206-3p, and hsa-miR-21-5p were upregulated in ALS CSF and serum, whereas hsa-miR-125a-5p was downregulated. Hsa-miR-206-3p and hsa-miR-210-3p correlated with disease duration and progression rate, while hsa-miR-21-5p correlated negatively with ALSFRS-R. No statistically significant results were found for hsa-miR-197-3p, between ALS phenotype and miRNA levels and between miRNA expression in CSF and serum. Functional enrichment analyses revealed biological signals related to cellular stress responses, TP53 signaling, SLIT/ROBO axonal guidance, innate immunity-like programs and tissue remodeling.

Conclusions

Serum and CSF hsa-miR-210-3p, hsa-miR-206-3p and hsa-miR-21-5p could serve as ALS biomarkers. The functional footprint of the dysregulated miRNAs suggest a stress-driven concept of ALS, involving axonal degeneration, immune activation and neuromuscular remodeling.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder of the central nervous system characterized by degeneration of upper and lower motor neurons, leading to muscle atrophy, paralysis and death, typically within a few years of onset [1, 2]. Despite major advances in neuroscience,

ALS remains a clinically heterogeneous disease with limited therapeutic options [3]. Reliable biomarkers for disease diagnosis, severity, and progression are urgently needed to improve diagnosis, phenotype stratification and monitoring in clinical settings [4].

MicroRNAs (miRNAs) are small non-coding RNAs (~ 22 nucleotides) that regulate post-transcriptional gene expression by binding to the 3' untranslated region (3' UTR) of target mRNAs, leading either to translational repression or mRNA degradation through the RNA-induced silencing complex (RISC) [5]. The miRNAs detected in cerebrospinal fluid (CSF) provide valuable information about the molecular processes that drive ALS, as they reflect both neuronal and glial pathophysiology and their contribution to disease progression [6, 7]. Their remarkable stability in CSF makes them attractive candidate biomarkers [8, 9].

For the present study, five miRNAs were selected, hsa-miR-206-3p, hsa-miR-210-3p, hsa-miR-197-3p, hsa-miR-125a-5p and hsa-miR-21-5p, which are involved in different but interconnected biological pathways, such as mitochondrial dysfunction, cellular stress, motor neuron apoptosis, neuroinflammation and neuromuscular junction disorganization [10–14]. The strategy of miRNAs selection was hypothesis-driven, aiming to link different biological mechanisms to ALS, while, simultaneously, filling the gap in bibliography concerning the representation of these miRNAs in ALS. Thus, hsa-miR-206-3p, was included, based on its established role as a muscle-specific miRNA (myomiR), implicated in molecular processes, such as denervation and regeneration of the neuromuscular junction [15]. This is one of the most frequently studied miRNAs in ALS, yet lacking experimental validation about its expression in the CSF [16, 17]. Hsa-miR-21-5p was selected, due to its association with cellular stress signaling, glial activation and neuroinflammation [18, 19]. Although this miRNA has been studied in ALS, mainly in murine models, research data remain ambiguous and, occasionally, conflicted, while connection with clinical parameters of ALS is underrepresented by the existing literature [20–22]. Hsa-miR-210-3p, a well-known miRNA related to hypoxia, cellular stress, response to DNA damage and mitochondrial dysfunction, is a biologically challenging, yet underexplored candidate in ALS [23–25]. Finally, for the other two miRNAs, hsa-miR-197-3p that is implicated in the regulation of apoptosis and neuroinflammation and hsa-miR-125a-5p that is involved in neuronal differentiation and endoplasmic reticulum stress, research evidence for their role in ALS is limited and scarce [26–28]. For each one of the five miRNAs, the most common arm strand that was encountered in bibliography was selected for our study.

The purpose of the present study is to detect reliable miRNA biomarkers for ALS diagnosis and/or disease progression and/or disease severity. In this view, we attempted to identify the expression profile of the selected miRNAs in the cerebrospinal fluid (CSF) and serum from ALS patients and compare it with the corresponding expression in the CSF and serum from non-ALS and healthy individuals. In addition, we endeavored to evaluate possible associations between the selected miRNA levels and the severity, duration, phenotype and progression rate of ALS patients and to interpret their biological significance through systematic pathway enrichment analysis of predicted target genes.

Materials and Methods

Study design and participant selection

This is a mono-centric, non-randomized, non-blinded, cross-sectional, case-control study. All participants were admitted to the Neurology Department, University Hospital of Larisa, Greece, from 2017 until 2024. Patients underwent detailed clinical and neurophysiological examination by two independent senior neurologists with expertise in neurodegenerative disorders. ALS diagnosis was based on the Revised El Escorial criteria [29]. Total blood was collected from all patients and was centrifuged until serum was extracted. Blood tests were conducted and included complete blood count and basic metabolic panel. Also, all patients underwent lumbar puncture and CSF was collected for miRNA analysis. The CSF and serum samples were stored at -80^{o}C. The ALS phenotype was defined by the site of disease onset as "bulbar", involving predominantly the degeneration of motor neurons located in the brainstem, with symptoms such as early dysarthria and/or dysphagia and/or tongue fasciculations, or "spinal" with onset, typically from the limbs [30]. The severity of the disease was evaluated with the Amyotrophic Lateral Sclerosis Functional Rating Scale – Revised (ALSFRS-R), disease duration was estimated in months and the disease progression rate (ΔFS) was calculated as (48 - ALSFRS-R)/disease duration).

Inclusion/exclusion criteria

In total, our study cohort consisted of 150 participants, 50 consecutive patients with definite ALS and 100 controls, 50 disease-controls and 50 healthy controls. ALS patients were eligible to take part in the study if they met the following criteria: i) definite ALS diagnosis, according to the Revised El Escorial criteria, ii) sample collection at the time of disease diagnosis, iii) age ≥ 18 years, iv) sporadic ALS forms, based on patients' family history, v) Greek origin, vi) complete disease history and clinical information, including age, symptoms at disease onset, disease duration until sample collection and ALSFRS-R at diagnosis, vii) blood tests with values within normal range, viii) no presence of comorbidities, such as active infections at sample collection or 4 weeks prior to sample collection, autoimmune disorders, malignancies or other neurodegenerative disorders, ix) adequacy of CSF and serum, without hemolysis, x) written informed consent.

The disease-control group for the CSF consisted of 50 (age > 18 years) participants, who underwent lumbar puncture only for routine diagnostic investigation. In detail, 14 patients with pseudotumor cerebri (PTC), 15 patients with normal pressure hydrocephalus (NPH) and 21 patients who admitted the hospital complaining about various symptoms, such as persistent headache and underwent lumbar puncture, but their examinations revealed no pathology. This disease-control group was age and sex matched to ALS patients. Serum and CSF were collected for miRNA analysis. Finally, 50 age and sex matched healthy controls (age > 18 years) were enrolled in this study and serum was collected. For the two control groups, the exclusion criteria were: presence of neurodegenerative disease, infection or malignancy and inadequate CSF and/or serum samples for RNA extraction. All participants provided informed consent

and the study was approved by the ethics committee of the University of Thessaly. The laboratory miRNA analysis and the statistical analysis were conducted by researchers blinded to the diagnosis and the demographic and clinical characteristics of the participants.

Study endpoints

The primary endpoint of our study was to evaluate whether the expression levels of the five selected miRNAs in CSF and serum could differentiate ALS patients from control subjects. Secondary endpoints included correlations of miRNA levels with clinical characteristics of the disease and, specifically, ALS phenotype, disease severity, disease duration and progression rate. Moreover, another secondary endpoint was the comparison of miRNA expression between CSF and serum. Finally, an exploratory endpoint of our study was to evaluate the biological and functional relevance of the most differentially expressed miRNAs through pathway enrichment analysis of their predicted target gene networks.

RNA extraction, miRNA quantification and real-time qPCR

Total RNA enriched for small RNAs was isolated from CSF and serum samples using Nucleozol, one phase RNA purification (Macherey-Nagel, Germany). The synthetic miRNA cel-miR-39-3p was used as spike-in control to monitor the extraction process [31]. The concentration of total RNA was calculated via NanoDrop microvolume spectrophotometer (Thermo Fischer Scientific, MA, USA) at a wavelength of 260nm and the ratio A260/A280 was used for purity check [32]. After the RNA extraction, hsa-miR-125b-5p was added as endogenous/reference control to check for technical variability [33]. Reverse transcription and cDNA synthesis was performed using Mir-X miRNA First-Strand Synthesis Kit (Clontech, Takara Bio Inc., Japan). Quantitative real-time PCR (qPCR) was performed in LightCycler 96, System (Roche, Basel, Switzerland) using FastGene IC Green (Nippon Genetics). Briefly, a mastermix containing 2x FastGene IC Green, 100ng cDNA, RNAse-free water and specific miRNA primers was prepared for each sample and for each miRNA, separately. For each miRNA, hsa-miR-206-3p, hsa-miR-210-3p, hsa-miR-197-3p, hsa-miR-125a-5p and hsa-miR-21-5p, a specific forward and a reverse primer were added in the mastermix. Each sample/miRNA was assayed in duplicate and quantified in the same qPCR reaction. Cyclic qPCR conditions involved a pre-incubation step at 95^{o}C, a 3-step amplification process (95^{o}C denaturation, 55-65^{o}C annealing and 72^{o}C extension) and a melting curve analysis step for specificity. Relative quantitative gene expression was calculated using the ΔΔCt method.

Ct values were obtained using uniform threshold settings. Ct values > 35 were excluded from the analysis, due to reduced validity. Average Ct values were used for each sample/miRNA. MiRNA expression levels were estimated for each sample and for each one of the five selected miRNAs as ΔCt, relative to reference miRNA, ΔCt(miRNA CSF/serum) = Ct(miRNA CSF/serum) – Ct(reference miRNA CSF/serum), with lower ΔCt values corresponding to higher expression. Differential expression between ALS and control groups was summarized as log2 fold change (log2FC), defined as − ΔΔCt, ΔΔCt = ΔCt(target) – ΔCt(control). Group-wise comparisons of miRNA expression between ALS patients and

control groups (healthy controls and disease controls) were performed separately for CSF and serum. Since ΔCt values were not assumed to be normally distributed, non-parametric tests were employed.

Differential expression and statistical analysis

For the statistical analysis, both IBM SPSS Statistics v28 and R Statistical Software (Version 4.1.2; R Core Team, 2021) were used. Primary group comparisons (ALS versus controls or disease-controls or disease-control subgroups) were conducted using the Mann-Whitney U test (for two groups) or the Kruskal-Wallis test (for more than two groups) using IBM SPSS Statistics v28. P-values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) procedure, through R Statistical Software. MiRNAs with FDR < 0.05 were considered statistically significant. The diagnostic performance of each miRNA was evaluated using logistic regression (dependent variable: ALS vs non-ALS) and receiver operating characteristic (ROC) curve analysis, with calculation of the area under the curve (AUC), with IBM SPSS Statistics v28.

Correlation with clinical variables

To assess whether the observed differences in miRNA expression were independent of demographic factors, ANOVA/ANCOVA models were applied through IBM SPSS Statistics v28, with ΔCt as the dependent variable and group (ALS vs disease-controls/controls), age and sex as independent variables. To evaluate whether miRNA expression was associated with clinical variables, correlations were computed between miRNA ΔCt values and ALSFRS-R scores, disease duration and progression rate (ΔFS) within the ALS group only. Spearman's rank correlation coefficient (ρ) was used through IBM SPSS Statistics v28, because it does not assume linearity or normality. Resulting p-values were corrected for multiple comparisons using FDR with R Statistical Software. Beyond simple correlations, multivariable linear regression models were applied with ALSFRS-R and progression rate as dependent variables and miRNA ΔCt values, age, and sex as independent variables, in order to determine whether the observed associations remained statistically significant after adjustment for confounding demographic factors. The overall fit (R^2 and adjusted R^2) and the independent contribution of each miRNA were estimated. In addition, the relationship between miRNAs and the speed of disease progression was evaluated, using logistic regression analyses. A dichotomized dependent variable of "fast" versus "slow progressors" was applied, using the median progression rate for dichotomization [34]. Thus, our cohort divided in 25 "fast progressors" and 25 "slow progressors". Logistic regression was used to estimate the probability of rapid progression as a direct consequence of miRNA levels, through the calculation of odds ratios. Both multivariate and linear regression analyses were performed through IBM SPSS Statistics v28. For the correlation of ALS phenotype with miRNA ΔCt, linear mixed-effects models were performed for each miRNA separately, using R Statistical Software. The two phenotypes (bulbar/spinal) were used as contrasts within CSF and serum and they were estimated using marginal means (emmeans). The p-values were corrected for multiple testing using the Benjamini–Hochberg FDR method. Equivalence testing using Two One-Sided Tests (TOST) on the mixed-model contrasts was also performed, with

predefined bounds (SESOI) of ±0.5 log2FC and 90% confidence intervals, along with sensitivity analyses with wider bounds of ± 1.5 log2FC.

Pathway enrichment analysis

After documenting both differential miRNA expression and associations with clinical severity and progression, the next step was to explore the functional meaning of this molecular profile. MiRNA-mRNA interactions were retrieved from integrated sources using the multiMiR package, allowing the combination of experimentally validated interactions with computationally predicted interactions.

Ranked gene lists were constructed for two contrasts: ALS versus healthy controls and ALS versus disease-controls. Enrichment was tested against three complementary databases: Reactome pathways, Gene Ontology Biological Process (GO BP) and Hallmark gene sets and was summarized using the normalized enrichment scores (NES), which reflects both the direction and magnitude of enrichment. Statistical significance was evaluated using permutation testing and adjusted for multiple comparisons using FDR. Gene sets with FDR < 0.05 were considered significantly enriched [35].

At the level of target gene lists, over-representation analysis (ORA) was performed on GO BP and Reactome, as well as on curated gene sets, where applicable. All enrichment results were interpreted using Benjamini-Hochberg FDR correction. In addition, preranked gene set enrichment analysis (GSEA) was applied to ranked lists of statistical scores [35]. This method provides normalized enrichment scores (NES) and adjusted p-values (FDR).

For independent evaluation of the consistency of the findings at the pathway level, an external transcriptomic dataset from Gene Expression Omnibus (GEO), (GSE155700) was used. Differential expression analysis was performed using DESeq2 on count data, based on a negative binomial model, followed by GSEA to the resulting ranked gene lists. The pathways derived from the miRNA target-based interpretation were compared with the pathways obtained from the independent transcriptomic dataset analysis, with emphasis on agreement at the gene set level and on the consistency of enrichment direction where interpretable. This external validation serves as independent evidence that reduces the risk of overinterpreting target-based results.

To test whether enrichment signals were stable and not contrast-dependent, the NES of the same pathways were compared directly between ALS vs disease-controls and ALS vs healthy. Positive NES indicated that a gene set shifts toward the ALS-associated end of the ranked list, while negative NES indicated the opposite. Higher |NES| showed stronger signal. Concordance was assessed by examining the consistency of both the sign and magnitude of NES values across contrasts, with particular emphasis on pathways showing significant enrichment in both analyses.

Results

Demographics

The study cohort consisted of 150 participants. In total, 50 ALS patients with mean age of 66.7 years ± 1.97 SD and mean disease duration of 9.68 months ± 2.796 SD, 50% females were enrolled in the study. The disease-control group included 50 age and sex-matched non-ALS patients, who underwent lumbar puncture only for diagnostic purposes and met the inclusion criteria for participation. Finally, 50 totally healthy age and sex matched controls, with mean age of 65.4 years ± 1.815 SD, were enrolled in our study. The main demographic characteristics of the study cohorts are listed in Table 1. The participant selection process with inclusion and exclusion criteria for patients and control groups are outlined at the flow diagram in **Online Resource 1**.

Table 1
Basic demographic characteristics of the study population at sample collection

Clinical characteristics	ALS patients	Disease-controls	Healthy controls
Sex (F/M)	25/25	25/25	25/25
Age (years ± SD)	66.7 ± 1.97	65.7 ± 2.296	65.4 ± 1.815
Phenotype (Spinal/Bulbar)	35/15		
Disease duration (months ± SD)	9.68 ± 2.796		
Amyotrophic Lateral Sclerosis Functional Rating Scale – Revised (ALSFRS-R) ± SD	32.18 ± 1.658		
Progression Rate (48-ALSFRS-R score/ disease-duration in months ± SD)	2.614 ± 0.504		

*Mean values ± Standard Deviation (SD) were calculated in 95% Confidence Interval (CI)

miRNA expression in the CSF and serum

In the CSF, clear differences in the expression of selected miRNAs were observed between ALS patients and disease-controls. Kruskal-Wallis test also confirmed the statistically significant differences among ALS and disease-control subgroups. Effect sizes were expressed as log2 fold change (log2FC) = $-\Delta\Delta Ct$, with negative values corresponding to lower expression in ALS (downregulation) and positive values to higher expression in ALS (upregulation). Specifically, hsa-miR-210-3p and hsa-miR-206-3p showed markedly increased expression in ALS, with positive log2FC values (approximately 3.43 and 3.03, respectively) and low FDR values (1.3×10^{-10} and 2.6×10^{-9}). Hsa-miR-21-5p also showed significantly increased expression in ALS (log2FC = 1.93, FDR 4.9×10^{-5}). In contrast, hsa-miR-125-5p showed reduced expression in ALS (negative log2FC = − 0.85) with borderline but acceptable significance after correction (FDR = 0.049). Hsa-miR-197-3p did not show a statistically significant difference (FDR = 0.46). MiRNAs differential expression is shown in Fig. 1.

The corresponding ΔCt distribution violin plots in **Online Resource 2**, visually confirm that miRNAs with positive log2FC display overall lower ΔCt values in ALS (thus higher expression), whereas miR-125a-5p shows the opposite pattern.

In the serum, the results were consistent regardless of the type of reference group (healthy controls or disease controls). Specifically, increased expression in ALS was observed for hsa-miR-206-3p, hsa-miR-21-5p, and hsa-miR-210-3p, with positive log2FC values and low FDR values both in the comparison of ALS vs healthy controls (log2FC = 3.27, 2.74, 3.39; FDR 8.5×10^{-11}, 1.0×10^{-7}, 4.7×10^{-13}, respectively) and ALS vs disease-controls (log2FC = 3.23, 2.47, 3.23; FDR 9.2×10^{-11}, 1.9×10^{-6}, 7.6×10^{-12}, respectively) and among disease-control subgroups. In contrast, hsa-miR-125-5p showed consistently reduced expression in ALS in both comparisons (log2FC = − 1.78 and − 1.75; FDR 1.6×10^{-5} and 2.3×10^{-5}). Hsa-miR-197-3p did not show statistically significant differences between ALS and healthy controls or between ALS and disease-controls (FDR 0.579 vs healthy and 0.453 vs disease-controls) or among ALS and disease-control subgroups. MiRNAs differential expression in the serum is exhibited in Fig. 2.

The ΔCt distributions in the serum are depicted in **Online Resource 3** for the groups ALS vs healthy controls and in **Online Resource 4** for ALS vs disease-controls.

No statistically significant differences were observed between CSF and serum across all samples and miRNAs. Assessment using the exogenous spike-in control cel-miR-39-3p showed stable recovery in both CSF and serum, with only small differences between groups. The endogenous control hsa-miR-125b-5p showed small Ct differences between ALS and control groups in both serum and CSF, on the order of approximately 0.3 Ct, with no statistical significance, supporting the adequacy of normalization.

Among all candidate biomarkers, hsa-miR-210-3p exhibited the strongest and most consistent diagnostic performance. In the serum, a significant difference was observed between ALS patients and controls, with an ROC AUC of 0.925, indicating excellent discriminative ability. In the CSF, miR-210-3p also showed a ΔCt difference of approximately three cycles between groups, with an AUC of about 0.902. In the serum, miR-206-3p showed significant overexpression in ALS patients with an AUC of 0.886. Similar findings were observed in the CSF, with an AUC of 0.866. Hsa-miR-21-5p showed clear overexpression in ALS patients in both serum and CSF, although its diagnostic performance was slightly lower than that of miR-210-3p and miR-206-3p, with values of 0.802 and 0.754, respectively. The ROC curves for the examined miRNAs are shown in **Online Resource 5**.

Association of miRNA expression with clinical variables

The correlation analysis revealed a particularly consistent pattern for hsa-miR-21-5p, which showed a strong negative correlation with ALSFRS-R in both serum and CSF, with FDR values (serum: $\rho=-0.82$, FDR = 1.5×10^{-11}; CSF: $\rho=-0.72$, FDR = 9.6×10^{-8}). In parallel, hsa-miR-206-3p and hsa-miR-210-3p showed consistent correlations with disease duration, with positive Spearman coefficients in both serum and CSF (CSF: $\rho = 0.56$, FDR = 2.2×10^{-4}; serum: $\rho = 0.57$ and 0.54, FDR = 1.9×10^{-4} and 3.2×10^{-4}, respectively). In addition, negative correlations with progression rate were observed for hsa-miR-206-3p and hsa-miR-

210-3p in CSF (ρ=−0.44, FDR = 0.006; ρ=−0.46, FDR = 0.005, respectively) and serum (ρ=−0.44, FDR = 0.006; ρ=−0.45, FDR = 0.005, respectively). In contrast, hsa-miR-197-3p, which did not differ systematically between groups, showed weaker and less consistent correlations. These patterns are exhibited in the correlation heatmap in Fig. 3.

Logistic regression models also exhibited that the diagnostic accuracy of the differentially expressed miRNAs was independent of age and sex as confounding factors and relied exclusively on the presence of the disease. Also, the comparison of miRNA expression between “fast” and “slow progressors” revealed no statistically significant differences for any of the examined miRNAs in CSF and serum, FDR values > 0.05. ΔCt mean values ± SD of the examined miRNAs for ‘fast’ and “slow progressors” are presented in **Online Resource 6**.

The correlation analysis of miRNA ΔCt with disease phenotypes, using linear mixed-effects models, did not reveal statistically significant differences in the expression of the five miRNAs between the bulbar and spinal phenotypes, either in the CSF or serum, after adjustment for age, sex, disease duration and ALSFRS-R and correction for multiple testing. The boxplots in **Online Resource 7** are consistent with this finding. Equivalence was not demonstrated through TOST for any of the studies miRNAs in either CSF or serum, not only for the ± 0.5 log2FC bound, but also for the wider bound of ± 1.5 log2FC.

The exogenous and endogenous controls (cel-miR-39-3p and hsa-miR-125b-5p) did not show strong or consistent clinical associations, reinforcing that the observed relationships are not due to technical variation.

Pathway enrichment analysis of miRNA targets

The functional interpretation was approached in two complementary directions: (a) targets of miRNAs increased in ALS represent genes likely to be suppressed in ALS, and (b) targets of the miRNAs decreased in ALS represent genes likely to show relative activation. This distinction allows enrichment analysis to indicate not only which pathways are involved, but also the likely direction of functional change.

Enrichment analysis using the Reactome database revealed a strong agreement of NES values over a wide range, with two clusters at positive and negative extremes, as shown in Fig. 4.

This indicates that many Reactome pathways showed consistent enrichment direction and magnitude regardless of reference group, suggesting that the functional signal reflects ALS-related shifts rather than peculiarities of the control group. Pathways labeled as replicated (min FDR ≤ 0.05 and max FDR ≤ 0.10 with the same NES direction) clustered at the extremes, indicating that the strongest signals are also the most reproducible. A recurring negative NES signal is seen for TP53/cell-cycle regulation, regulation of SLIT/ROBO expression and host–virus/innate immunity-like programs. An additional positive signal for PTK6 signaling was observed in the ALS vs healthy comparison, a finding that is not evident when the control group included disease-controls. These results indicate coherent functional shifts related to stress/survival signaling, axon guidance and neuronal architecture (SLIT/ROBO), and

innate immune/stress programs. The Reactome GSEA of predicted miRNA targets for ALS patients vs diasease-controls and for ALS patients vs healthy controls is shown in Fig. 5.

Analysis using the Hallmark gene set collection identified a single, highly robust and reproducible signal across both contrasts, at the gene set named HALLMARK_UV_RESPONSE_DN, **Online Resource 8**. This gene set captures a generalized cellular stress-response program associated with DNA damage, oxidative stress, and proteostatic imbalance. The consistency of this signal across both ALS versus healthy and ALS versus disease-control contrasts indicates a stable cellular stress-response signature in ALS.

GO Biological Process analysis further refined the functional interpretation by revealing significant enrichment of terms related to morphogenesis and tissue remodeling. Our analysis showed strong signals in morphogenesis/development-related processes and regulation of muscle differentiation, consistent with neuromuscular remodeling and stress-related metabolic reprogramming in ALS. In contrast, gene sets related to nucleobase and nucleotide catabolism showed opposite directionality, indicating metabolic reprogramming under conditions of sustained cellular stress. GO BP GSEA of predicted miRNA targets for ALS patients vs disease-controls are depicted in Fig. 6.

The chord plot depicting the predicted gene targets of miRNAs and their relation to biological processes through GO BP for ALS patients vs disease-controls is provided in **Online Resource 9**.

Discussion

In the present study, we quantified the expression levels of hsa-miR-206-3p, hsa-miR-210-3p, hsa-miR-197-3p, hsa-miR-125a-5p and hsa-miR-21-5p in CSF and serum from ALS patients and disease-controls, and in the serum of healthy individuals, through qRT-PCR. Expression levels for each miRNA were presented in relation to reference miRNA, as ΔCt. Comparisons of miRNA expression levels between ALS patients and control groups were performed to assess the diagnostic performance of the selected miRNAs. Within the ALS group, correlations of miRNAs ΔCt with disease phenotype, ALSFRS-R, disease duration and progression rate were conducted. The most dysregulated miRNAs from our study were used for pathway enrichment analysis of predicted target genes with external validation in independent transcriptomic dataset.

Overall, miRNA expression revealed a consistent and biologically coherent pattern. In both cerebrospinal fluid and serum, three miRNAs: hsa-miR-210-3p, hsa-miR-206-3p, and hsa-miR-21-5p were upregulated in ALS, whereas hsa-miR-125a-5p was downregulated and hsa-miR-197-3p showed no differential expression, effectively functioning as a negative specificity control. Consistency of these findings, particularly in serum, against both healthy and disease-controls, supports the interpretation that the observed signal is not driven by a specific control group but reflects a disease-related molecular signature.

In terms of clinical correlations, the most prominent association, with very low FDR values, concerned hsa-miR-21-5p levels and ALSFRS-R in both CSF and serum. The correlation was negative, meaning that as hsa-miR-21-5p ΔCt decreased, also decreased ALSFRS-R score. Since lower ΔCt values correspond to higher miRNA expression levels, this finding suggests that higher hsa-miR-21-5p expression levels are linked to greater disease severity. Similarly, hsa-miR-206-3p and hsa-miR-210-3p were found to be associated with disease duration and progression rate. Both demonstrated positive correlations between disease duration and ΔCt values, indicating a tendency towards lower miRNA levels with disease progression and negative correlations between ΔCt values and progression rate, suggesting higher miRNA expression levels in faster progressive disease. The important point about our findings is that the miRNAs that exhibited the best diagnostic performance, have also been found to be clinically associated to ALS, opposing a chance statistically significant finding.

Our results are in accordance with previous studies demonstrating increased levels of hsa-miR-206-3p in the serum of ALS patients [36, 37]. In contrast to studies linking upregulation of miR-206 to slow disease progression, in our study hsa-miR-206-3p ΔCt showed a negative correlation with progression rate [34, 38, 39]. Since miR-206 is involved in neuromuscular junction repair and compensatory response to motor neuron degeneration, our finding could possibly suggest that faster disease progression is related to stronger denervation and thus, stronger compensatory response and upregulation of miR-206 [15, 40].

As for miR-21, Joilin et al., 2020, showed that miR-21-5p was reduced in the serum of ALS patients [22]. On the other hand, Cuhna et.al., 2018, reported upregulation of miR-21-5p in the lumbar spinal cord of $SOD1^{G93A}$ mice, while Gomez et.al., 2020, exhibited elevated expression levels of miR-21 in spinal cord-derived astrocytes and reduced miR-21 levels in cortical astrocytes from transgenic $SOD1^{G93A}$ mice, indicating regional heterogeneity [20, 21]. Since miR-21 is mainly involved in the regulation of immune reactions of astrocytes and microglia, the differential expression of this miRNA may be probably dependent of the affected region and the immune state [18]. In our study, hsa-miR-21-5p was found to be upregulated in the CSF and serum from ALS patients and associated with their functional status.

Based on our results concerning miRNA expression, pathway enrichment analysis was performed by using predicted miRNA targets. In our study, the miRNA profile was predominantly upregulated in ALS. Since miRNAs generally act as negative regulators of their targets, enrichment analysis described which pathways are most likely to be dysregulated through miRNA target suppression [41, 42].

Reactome GSEA results showed a repetitive pattern, characterized by negative NES for pathways related to TP53-mediated cell-cycle regulation, SLIT/ROBO signaling of neuronal axons and host-virus host–virus/innate immunity-like interaction networks. The directionality remained stable across both contrasts, healthy and disease-controls, suggesting a consistent functional interpretation. As for the biological processes that encompass these pathways, they involve regulation of DNA damage responses, control of cell-cycle and survival versus apoptosis decisions, neuronal connectivity and axonal maintenance [43–45]. Also, the enriched pathways within host–virus interaction and innate

immunity-like categories can be better interpreted as indicators of cellular stress and inflammatory activation, rather than viral infection involvement [46, 47].

In contrast, positive NES concerning PTK6 signaling emerged only in the comparison between ALS and healthy controls, suggesting that this signal can be more sensitive to this specific reference group. The distinction between replicated and contrast-sensitive findings implies that replicated results may represent more stable biological signatures, whereas contrast-dependent results require cautious interpretation and further validation [48, 49].

The Hallmark GSEA showed a more unified signal. The only statistically significant term with positive NES was HALLMARK_UV_RESPONSE_DN, which remained stable across both reference groups. The functional interpretation of this gene set is that it represents a regulator of cellular stress and DNA damage response that is totally replicated [50]. This finding does not contradict the Reactome results. It summarizes them at a higher level of biological abstraction, where Hallmark reflects the overarching theme and Reactome delineates the underlying mechanistic pathways.

Finally, the GO Biological Process analysis highlighted terms related to morphogenesis and developmental programs in the direction of positive enrichment, while processes involving nucleobase and nucleotide catabolism appeared in the opposite direction. These findings should not be interpreted as evidence of “developmental activation” in ALS, but rather as an indication that the targets of differentially expressed and clinically associated miRNAs cluster within functional categories linked to tissue remodeling, cellular reprogramming and nucleic acid–related metabolic processes [51]. Such a pattern is consistent with a complex neurodegenerative condition characterized by neuronal injury, compensatory responses and cellular stress [52].

In summary, while most of these miRNAs, particularly has-miR-206-3p and has-miR-21-5p, have previously been studied in ALS, our study provides novel evidence in terms of examining these miRNAs together, across CSF and serum, with clinical correlations and functional interpretation through pathway enrichment analysis [7, 53, 54]. The strength of our findings arises from convergence across independent fields: stable miRNA signals in both serum and CSF, consistent associations with clinical variables, reproducible pathway-level patterns across Reactome analyses with complementary confirmation in Hallmark signatures and sensitivity testing with respect to control group selection. Together, these elements support the presence of a coherent molecular signature linking miRNA regulation to core pathobiological mechanisms of ALS.

Limitations

Despite the robust findings, our study holds several limitations that should be taken into consideration. First of all, although the cohort that was enrolled in this study was sufficient to detect large effect sizes, the sample size may still be underpowered to identify smaller effect sizes and/or subtle clinical correlations [55]. Second, a panel of five selected miRNAs was used in this study and miRNA expression levels were validated with qRT-PCR. While this method is highly sensitive and accurate, the findings are

restricted to the selected candidates and does not offer a broader perspective of dysregulated miRNAs, as with microarrays or small RNA sequencing. Studies using genome-wide miRNA profiling are needed to reveal novel ALS-associated miRNAs [56]. Third, two biofluids, CSF and serum, were analyzed for miRNA expression in our study and therefore, our results cannot be generalized and trends in miRNA profiles concerning other materials, such as plasma, tissues, peripheral blood cells or exosomes cannot be represented [57]. Fourth, the design of our study was cross-sectional, providing clinical data and miRNA correlations at a single point in time, set at patients' diagnosis. Although correlations with disease duration and progression rate were conducted, longitudinal sample collection and miRNA correlations with clinical parameters over time could provide more reliable results on how these miRNAs interfere with causality and disease prognosis [38]. Fifth, pathway enrichment analysis was based on predicted miRNA targets. While these targets are validated and are considered highly confident, the identified interactions remain probabilistic and limited. Further experimental validation is necessary to confirm the functional associations and the implicated pathways [58]. Also, another methodological limitation concerns normalization. The lack of universally stable miRNAs across biofluids that can be used as endogenous controls, remains a challenge [59]. In our study, endogenous and spike-in controls were applied to reduce technical variability. Although hsa-miR-125b-5p that was used as endogenous reference remained relatively stable in our samples and did not show any statistically significant differences, residual bias cannot be ruled out. Further studies, using multiple reference miRNAs and/or global mean normalization would strengthen the results [60]. Furthermore, since the control cohort included disease-controls, heterogeneity within this group may have influenced the findings. Validation in larger cohorts with more homogeneous control populations is therefore necessary to confirm the diagnostic utility of these results, as well as their clinical correlations and functional relevance [61]. Moreover, genetic analysis of our patients was not performed, thus, preventing stratification of ALS cohort according to mutations that may influence miRNA expression or disease heterogeneity [62]. Finally, data concerning environmental exposure, such as pesticides and/or heavy metals, as well as information on lifestyle, were limited, inhibiting possible correlation with miRNA profiles in our study. Further studies, integrating the genetic and environmental background of participants would strengthen the diagnostic accuracy and the biological significance of candidate biomarkers [63, 64].

Conclusions

The present study underlines the significance of a panel of circulating miRNAs in the cerebrospinal fluid and serum as a molecular signature in ALS. In particular, hsa-miR-210-3p and hsa-miR-206-3p could serve as robust diagnostic biomarkers, while hsa-miR-21-5p can be used as a biomarker of disease severity. Additionally, the downregulation of hsa-miR-125a-5p in ALS patients further supports the concept that the combination of upregulated and downregulated miRNAs, with diagnostic performance and/or clinical correlations could be used as a multimarker panel for ALS diagnosis and disease monitoring.

Functional analyses of enriched pathways for the most dysregulated miRNAs of our study indicated that the signals produced by the predicted target gene sets converged on molecular mechanisms that involve

responses to cellular stress and DNA damage, pathways related to axonal integrity, immune-like activation networks and tissue remodeling. This pattern is in concordance with current perspectives that describe ALS as a multifactorial neurodegenerative disorder with distinct pathophysiology linked to chronic cellular stress, disrupted neuronal integrity and compensatory responses. Further experimental research with longitudinal sampling is essential to establish causal associations and evaluate the potential of these miRNAs as biomarkers of disease prognosis and targeted therapy in ALS.

Declarations

Conflicts of interest

The authors have no competing interests to declare that are relevant to the content of this article.

Ethics approval

The study protocol has been approved by the ethics committee of the University of Thessaly and it is in line with the Declaration of Helsinki. All participants signed informed consent for personal data and sample collection and for publication of the study results.

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Author Contribution

All authors contributed to the study conception and design. Patient selection and clinical evaluation was performed by Polyxeni Stamati and Efthimios Dardiotis. Sample collection was performed by Maria Sokratous and Chrysoula Marogiani. MiRNA analysis was conducted by Panagiotis Liakos, Dimitrios Bogdanos and Maria Sokratous. Pathway enrichment analysis was conducted by Maria Sokratous and Polyxeni Stamati. Statistical analysis was performed by Ioannis Liampas and Vasileios Siokas. All authors read and approved the final manuscript.

Data availability

The data that support the findings of the study will be available upon reasonable request from the corresponding author.

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Figures

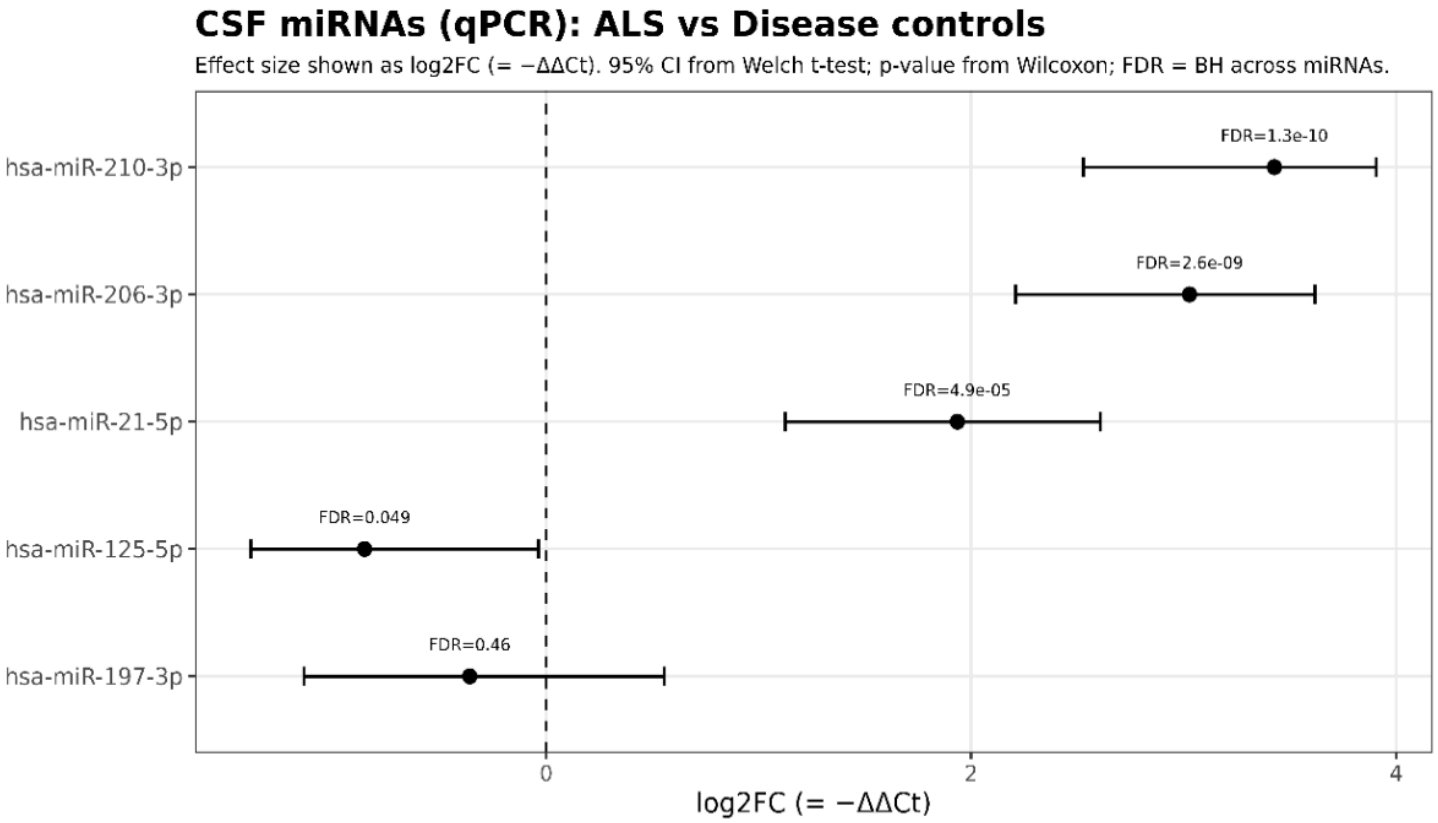


Figure 1

(CSF) Comparison of miRNA expression in CSF: ALS vs disease-controls. Effect size is shown as log2FC (= −ΔΔCt). The 95% confidence intervals were calculated using Welch's t-test, while p-values were obtained from the non-parametric Wilcoxon test. Multiple-comparison correction was applied using Benjamini–Hochberg (FDR) at the miRNA level.

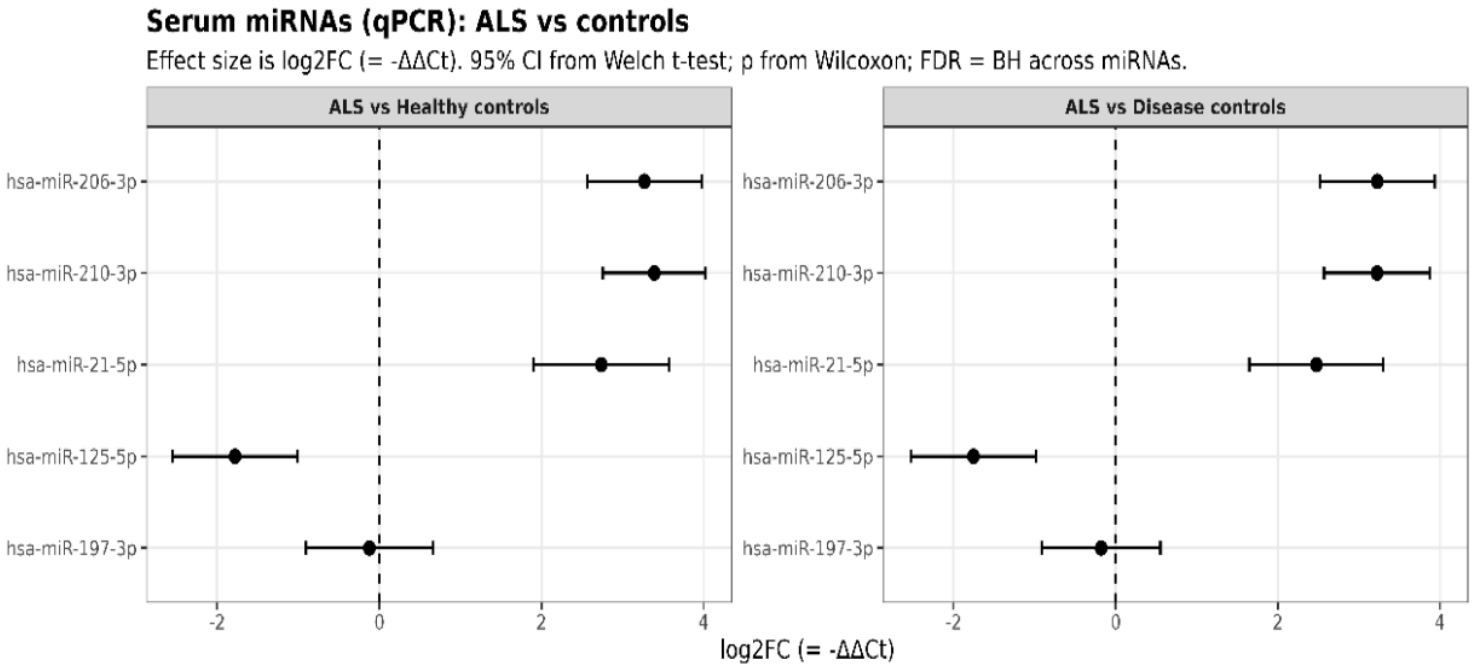


Figure 2

(Serum) Comparison of miRNA expression in serum: ALS vs healthy controls and ALS vs disease-controls. Effect size is shown as log2FC (= −ΔΔCt). The 95% confidence intervals are derived from Welch's t-test, p-values from the non-parametric Wilcoxon test, and multiple-comparison correction was applied using Benjamini–Hochberg (FDR) at the miRNA level.

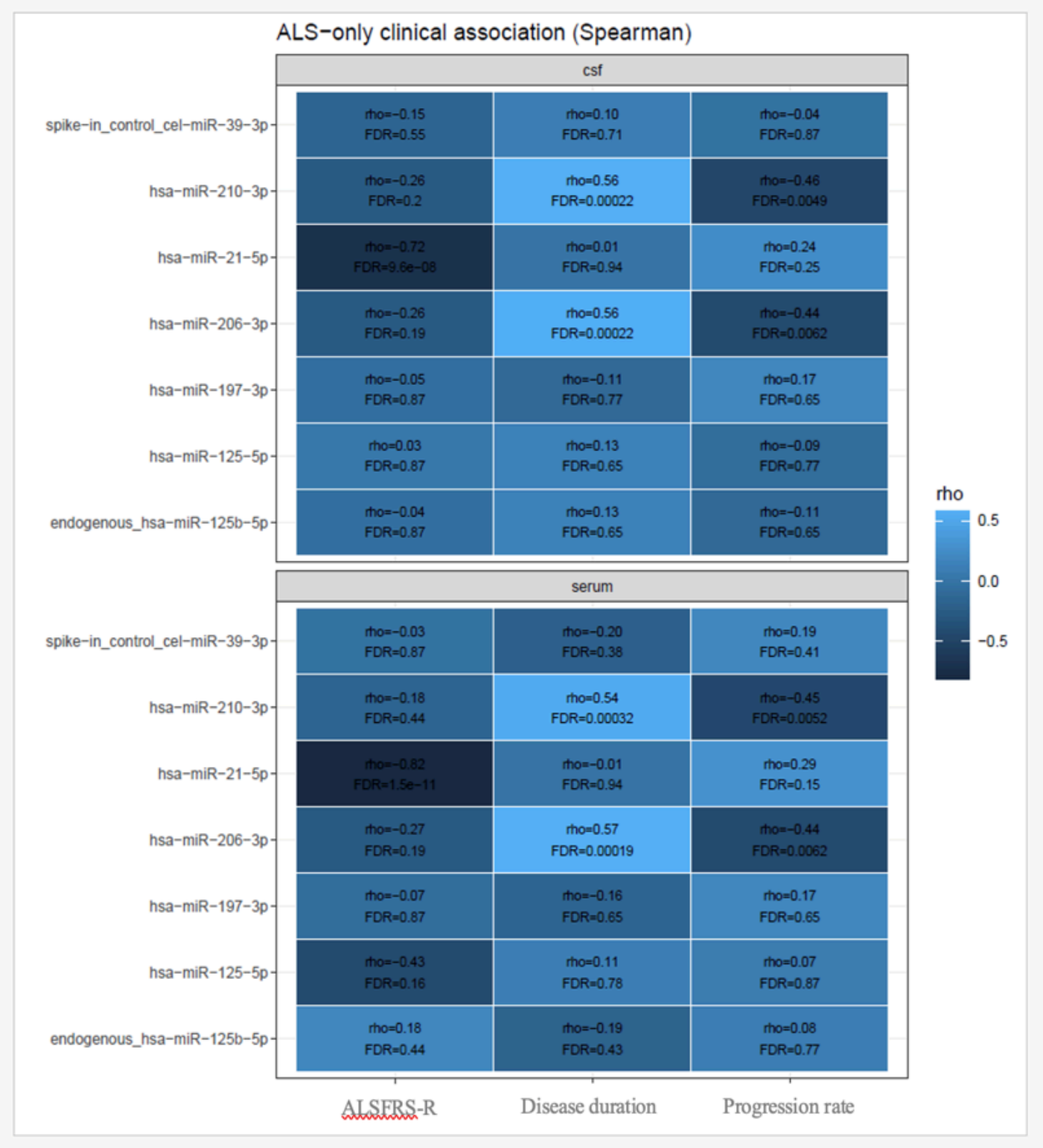


Figure 3

Heatmap of correlations (Spearman ρ) between ΔCt values of selected miRNAs and clinical variables within the ALS group, separately for serum and CSF. Shown are correlations with ALSFRS-R, disease duration (months), and progression rate (ΔFS). Each cell includes Spearman's ρ and the corresponding FDR-corrected p-value (Benjamini–Hochberg). Lower ΔCt corresponds to higher expression, so the sign of ρ is interpreted accordingly.

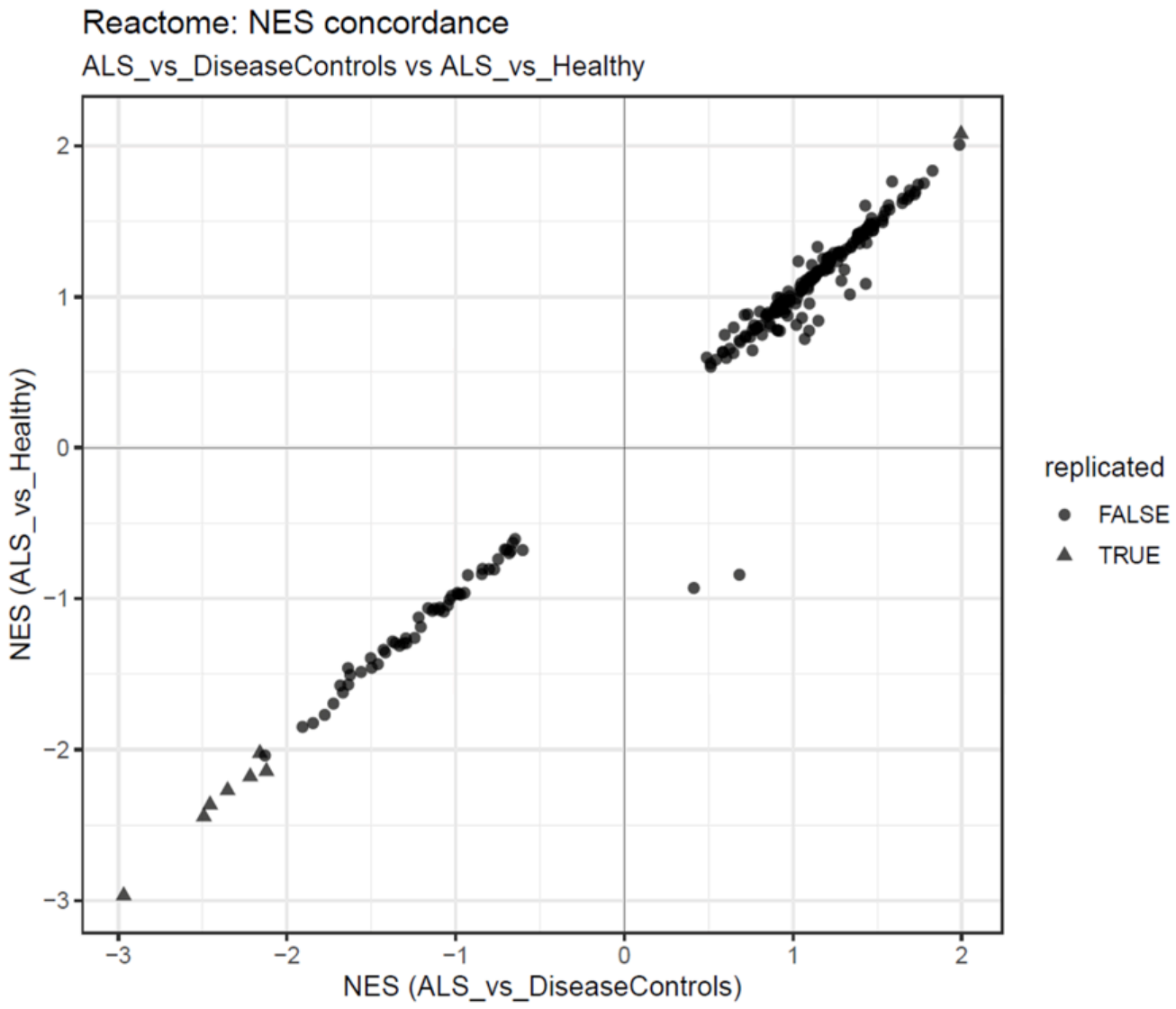


Figure 4

Reactome NES concordance between two contrasts. Scatter plot of NES values for Reactome pathways in ALS vs Disease-Controls (x-axis) versus ALS vs Healthy (y-axis). Each point represents a pathway. Proximity to the diagonal indicates agreement in direction and magnitude. Replicated pathways (criterion: min FDR ≤0.05 and max FDR ≤0.10 with same NES direction) are highlighted.

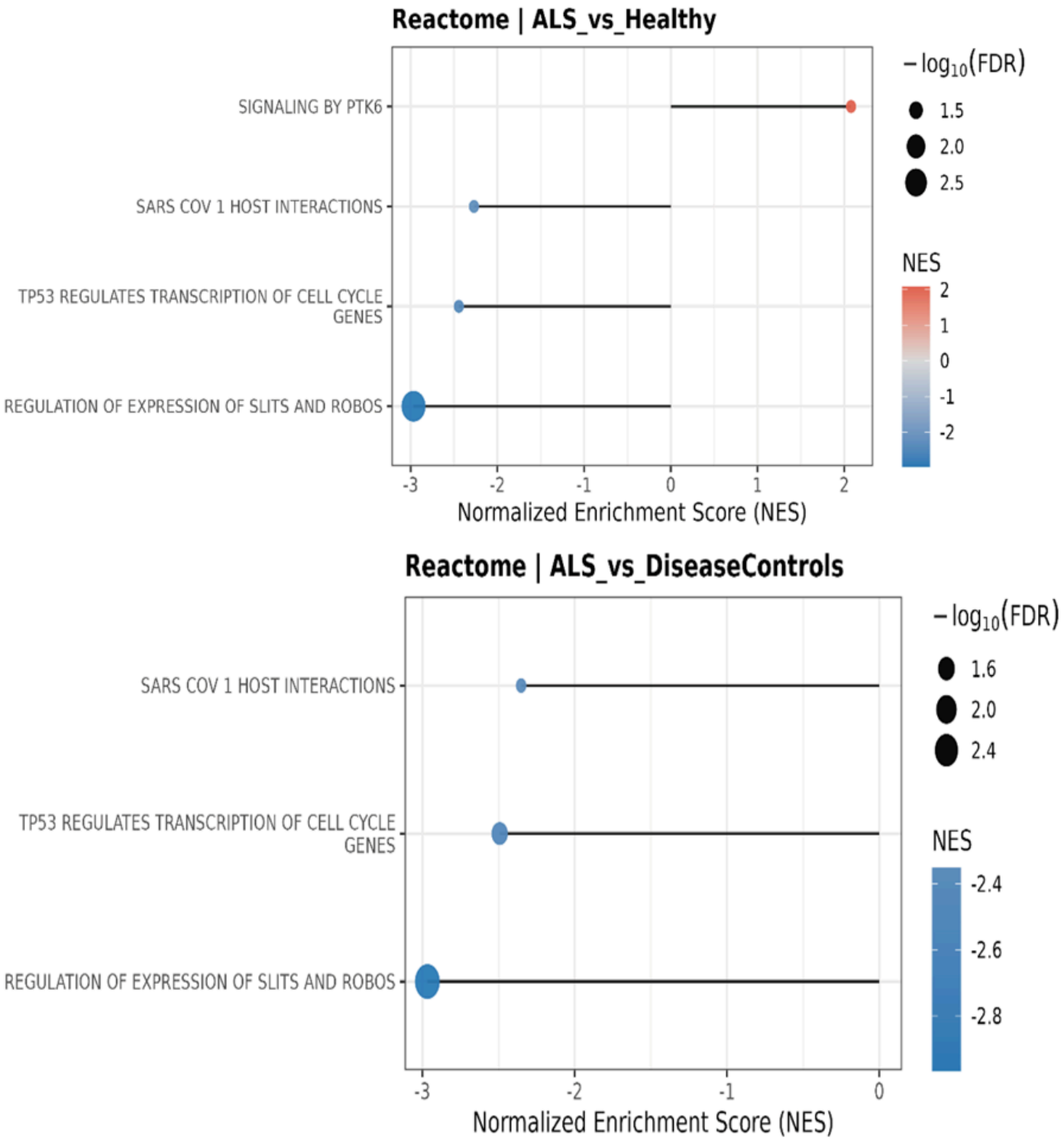


Figure 5

Reactome GSEA of predicted miRNA targets in ALS in two contrasts. Significant Reactome pathways (FDR<0.05, strict/reduced) are shown for ALS vs Healthy (top) and ALS vs Disease-Controls (bottom). The x-axis corresponds to NES; point size reflects −log10(FDR). A recurring negative NES signal is seen for TP53/cell-cycle regulation, regulation of SLIT/ROBO expression, and host–virus/innate immunity-like programs, with an additional positive signal for PTK6 signaling in ALS vs Healthy.

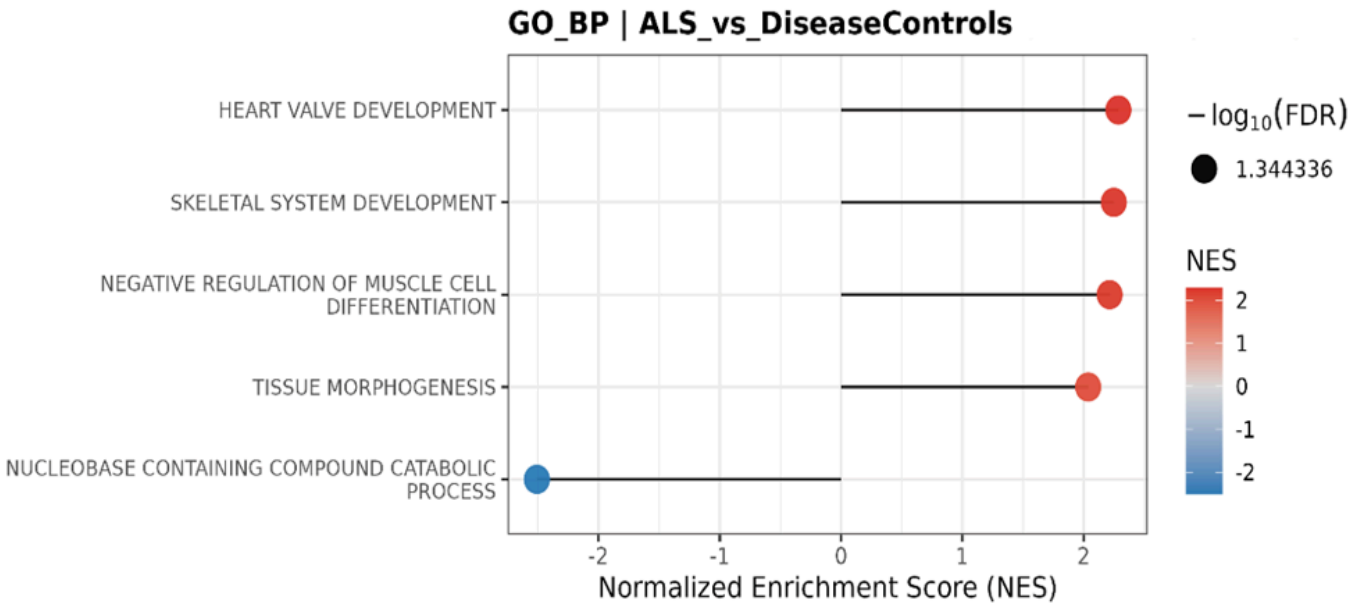


Figure 6

GO BP GSEA of predicted miRNA targets (FDR<0.05). Results show strong signals in morphogenesis/development-related processes and regulation of muscle differentiation, consistent with neuromuscular remodeling and stress-related metabolic reprogramming in ALS.

Supplementary Files

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