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MYOMIRNAS ROLE IN ALS SUGGEST A CROSSTALK BETWEEN MUSCLE AND MOTOR NEURON

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ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a rare, progressive, neurodegenerative disorder caused by degeneration of upper and lower motor neurons. The disease process leads from lower motor neuron involvement to progressive muscle atrophy, weakness, fasciculations for the upper motor neuron involvement to spasticity. Muscle atrophy in ALS is caused by a dysregulation in the molecular network controlling fast and slow muscle fibres. Denervation and reinnervation processes in skeletal muscle occur in the course of ALS and are modulated by rehabilitation.

MicroRNAs (miRNAs) are small non-coding RNAs that modulate a wide range of biological functions under various pathophysiological conditions. MiRNAs can be secreted by various cell types and they are markedly stable in body fluids. MiR-1, miR-133 a, miR-133b, and miR-206 are called “myomiRs” and are considered markers of myogenesis during muscle regeneration and neuromuscular junction stabilization or sprouting.

We observed a positive effect of a standard aerobic exercise rehabilitative protocol conducted for six weeks in 18 ALS patients during hospitalization in our center. We correlated clinical scales with molecular data on myomiRs. After six weeks of moderate aerobic exercise, myomiRNAs were down-regulated, suggesting an active proliferation of satellite cells in muscle and increased neuromuscular junctions. Our data suggest that circulating miRNAs modulate during skeletal muscle recovery in response to physical rehabilitation in ALS.

Keywords: ALS, ALS rehabilitation, myomiRs, circulating miRNAs, muscle, motor neuron.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a rare, progressive, neurodegenerative disease that involves lower motor neurons in the spinal cord or brainstem and upper motor neurons in the motor cortex. Loss of motor neurons leads to muscle atrophy and weakness, fasciculations, and spasticity [1]. Approximately two-thirds of patients with ALS have the classical 'spinal form' of the disease [2] with onset in lower limb and symptoms associated with muscle atrophy. Patients with a bulbar onset of motor neuron disease (MND) initially exhibit dysarthria and dysphagia for solids and liquids [2]. In MND death occurs within 3-5 years but there is a great variability in the duration of the disease since few patients die a few months after the onset and others survive for more than twenty years [1].

The causes of sporadic ALS probably occur as a result of complex interactions between environmental mechanisms and the activation of ALS pathogenetic mechanisms such as oxidative stress, defects in RNA processes, mitochondrial dysfunction, protein aggregates, excitotoxicity, problems in axonal transport, and inflammation [3–5] Pathogenic processes leading to the disease involve both motorneurons and non-neuronal cells including astrocytes, microglia, T-cells, and skeletal muscle. Muscle atrophy in ALS is caused by a disregulation in the 'molecular network' of autophagy, mitochondrial biogenesis, the proliferation of satellite cells, and muscle regeneration processes [3,4]. Therefore, structural and metabolic changes in skeletal muscle can aggravate the course of the disease. Recent studies suggest that skeletal muscle contributes to a retrograde signaling cascade that impairs motor neurons [6–8].

In a trial [9] we found that regular, rehabilitative exercise in ALS patients helps to reduce pain and fatigue of skeletal muscle origin. If a patient is inactive, the loss of training and disuse leads to muscular atrophy, which adds to the weakness and muscular atrophy caused by denervation and degeneration of motor neurons in ALS.

MicroRNAs (miRNAs) are small, noncoding, single-stranded RNA (19-24 nucleotide) molecules, highly conserved during evolution, which possess a high specificity of tissue during various stages of development. They negatively regulate gene expression at the post-transcriptional level [10] by pairing with specific messenger RNAs (mRNAs), leading to degradation or preventing translation into the corresponding protein product. The up-regulation of a specific miRNA determines a decrease in the expression of the corresponding protein product. Bioinformatics predictions indicate that mammalian miRNAs could regulate more than 30% of all proteins encoded by genes [11].

MiRNAs are involved in a wide range of physiological and pathological processes, including muscle, and their dysregulation is involved in several human diseases. Evidence has highlighted the role of miRNAs and their abnormal expression in neurodegenerative diseases. Dysregulation of miRNAs has been documented in ALS [12–14], Alzheimer, Huntington and Parkinson diseases [15,16]. MiRNAs are promising potential diagnostic biomarkers [17] and they could be utilized to monitor the progression of the disease and to evaluate response to pharmacological or rehabilitative treatments.

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In this study, we correlate observation on the effects of physical rehabilitative practice in ALS patients with molecular data on serum muscle-specific miRNAs as biomarkers of muscle regeneration and neuromuscular-junctions recovery.

Materials and Methods

Patient selection

A cohort of 18 ALS patients was included in this study and diagnosed according to the revised El Escorial criteria [18]. Inclusion criteria included: a sporadic ALS form, mild to moderate disability where the patient could walk without an assisted device. We excluded ALS patients with: a genetic form of ALS, cardiac involvement, history of other neurological and metabolic disorders, and severe neuropsychiatric illness that caused patients to be unable to understand and perform instructions. The level of physical disability was assessed using the revised ALS functional rating scale (ALSFR-R).

The study was performed in accordance with the ethical standards of the Declaration of Helsinki. The investigation and use of patients' data for research purposes were approved by the local research ethics committee in accordance with the Declaration of the World Medical Association.

Serum samples were obtained from peripheral blood of 18 ALS patients after written informed consent. In ALS patients serum was collected at the beginning of the patient's admission, hereafter called Time Zero (T0, baseline), and the second after a period of physical rehabilitation named Time One (T1). Biological samples were stored frozen at -80°C in Biobank of Rare Diseases and Neuro-rehabilitation (BBMRNR) at Foundation Hospital San Camillo IRCCS until use.

Patient rehabilitation protocol

The rehabilitation programme consisted of an individualized progressive training of muscular strengthening and aerobic endurance exercises to avoid muscle damage, performed daily for 6 weeks during hospitalization. In relation to the disability the ALS patients performed a cycle ergometer, ergometry arm-leg and/ or treadmill or a standard rehabilitation consisting of a one-hour session of stretching, active mobilization, and general reinforcement as previously described [9]. In addition, all ALS patients underwent speech, occupational, and psychological therapy.

A series of clinical scales was assessed before and after physical rehabilitation: functional autonomy was evaluated by the Functional Independence Measure (FIM). The Fatigue Severity Scale (FSS) was administered to measure the degree of fatigue and its effect on the patient's activities. The Barthel Index was used to measure performance improvement in daily life activities.

RNA extraction and miRNA quantification

MiRNAs were isolated from serum using the miRNA easy Mini Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. RNA was reverse transcribed using TaqMan microRNAs reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) according to manufacturer's protocol and specific probes for each miRNA (miR-1, miR-133a, miR-133b, and miR-206) were used. The resulting cDNA was amplified by Real-Time PCR using TaqMan microRNA assay primers.
MiRNA levels were calculated using the ΔΔCT method. MiRNA expression levels were normalized using miR-39-3p of C. elegans as previously described [13,19] added as a spike-in control to measure the efficiency of RNA extraction, reverse transcription, and PCR amplification. Baseline data, Time Zero (T0) before rehabilitation treatment, were set as a control to calculate fold change using the 2-ΔΔCt method of Time One (T1).

Statistical analysis

We used the Wilcoxon-Mann-Whitney test for paired data for small samples to verify the validity of data obtained. The level of significance was set at $p < 0.05$. In the graphs, values are expressed as a mean ± standard deviation. Data were analysed using the R-studio program for Windows

Results

Patient cohort and selection

Eighteen patients were selected from a cohort of twenty-one ALS patients admitted to Foundation Hospital San Camillo IRCCS (Venice, Italy) and were evaluated in the course of 6 weeks of physical rehabilitation. Eighteen patients who met the inclusion criteria, described in the materials and methods were enrolled in the study. Patients with a genetic form of the disease were excluded. The clinical features of ALS patients are reported in Table 1. The ALS patients (eleven male and seven female), had an average age of 61.1 years with a mild or moderate disability with an ALSFR-R average mean of 34.6±4.9.

TABLE 1: Clinical characteristics of the ALS patients at baseline

ALS features	Mean±SD
Age (years)	61.1±12.8
Sex M/F	11/7
Disease duration (years)	4.3±3
ALSFRS-R	34.6±4.9

ALSFRS-R: Amyotrophic Lateral Sclerosis Functional Scale-revised

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We collected clinical scales and peripheral blood from selected ALS patients before (T0) and after (T1) a period of six weeks of training.

TABLE 2 Clinical features and disability scales of ALS patients before (T0) and after (T1) rehabilitation treatment

Patient	Age	Sex	ALSFS-R T0	ALSFS-R T1	FSS T0	FSS T1	FIM T0	FIM T1	Barthel T0	Barthel T1
Pt 01	74	M	29	29	5,40	3,90	78	80	45	60
Pt 02	51	M	40	41	5,5	3,9	77	82	50	55
Pt 03	57	F	38	40	5,60	4,20	86	90	55	70
Pt 04	79	M	29	29	5,30	5,20	85	87	30	40
Pt 05	50	M	40	41	5,50	3,90	77	82	50	55
Pt 06	61	M	31	31	5,50	5,50	37	36	N.A.	N.A.
Pt 07	45	F	28	28	5,50	5,50	88	91	70	80
Pt 08	33	F	32	32	5,20	4,80	65	69	35	40
Pt 09	76	F	34	34	5,50	4,90	N.A.	65	20	35
Pt 10	44	M	32	32	5,40	5,00	83	87	60	65
Pt 11	71	M	36	36	5,50	5,10	N.A.	69	20	35
Pt 12	71	M	32	33	5,40	3,90	78	84	45	55
Pt 13	61	F	33	34	5,10	4,00	60	70	35	45
Pt 14	71	M	28	28	5,60	4,40	65	N.A.	40	45
Pt 15	65	F	39	40	4,50	3,20	83	85	50	55
Pt 16	65	F	42	43	5,40	4,20	73	79	45	50
Pt 17	72	M	36	36	5,60	4,00	81	89	60	90
Pt 18	54	M	43	44	4,80	3,20	80	88	60	70

ALSFS-R: Amyotrophic Lateral Sclerosis Functional Scale-revised; FSS: Fatigue Severity Scale; FIM: Functional Independence Measure. N.A.: not available

In Table 2 we report sex, age, and the values of clinical outcome, according to scale measurement recorded at T0 and at T1 for every ALS patient. After physical training, the ALS patients showed an improvement of muscle strength, an improvement of physical conditions, and independence documented by a significant change (p-value≤0.05) in ALSFR-R, Barthel and FIM scores and a decreased sense of fatigue registered by FSS in T1 (p-value≤0.05) underlining the positive effect of rehabilitation.

Circulating miRNA expression

We measured circulating muscle-specific miRNAs by qRT-PCR in the serum of eighteen sporadic ALS patients before (T0) and after (T1) 6 weeks of physical rehabilitation. We found a significant down-regulation of miR-1, miR-206, miR-133a, and miR-133b as shown in Fig 1. We believe that the decrease in expression levels of myomiRs that we found after training try to restore a normal biological condition in skeletal muscle and neuromuscular junction (NMJ) of ALS patients.

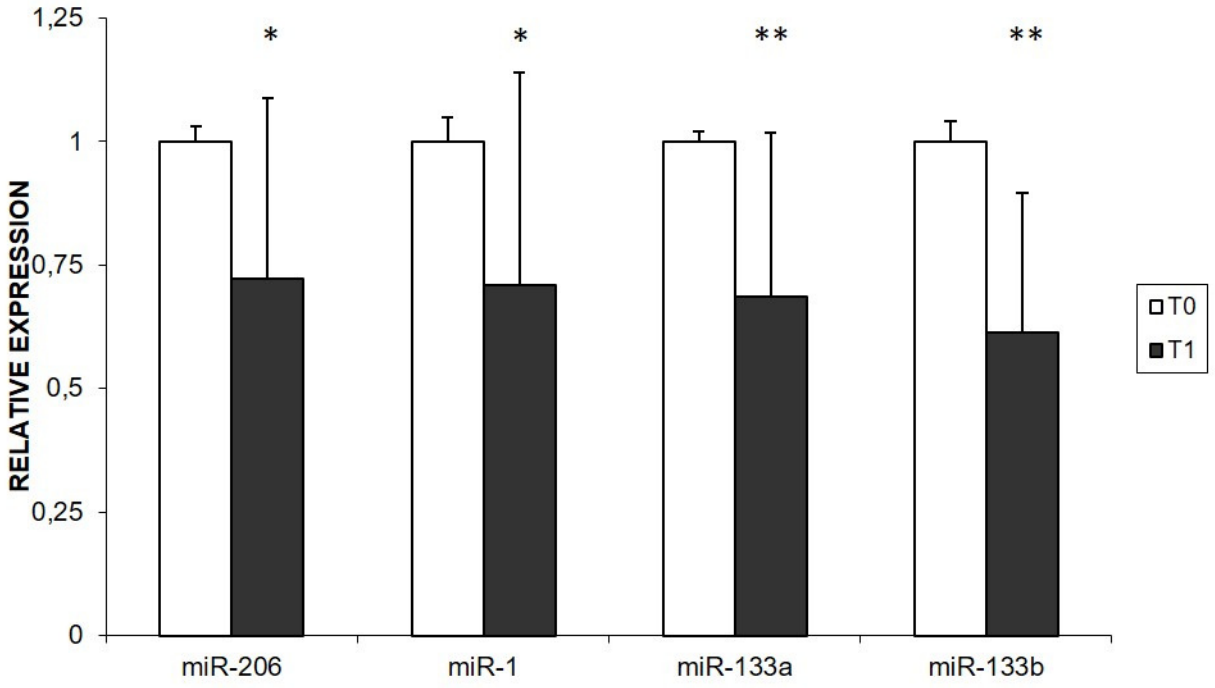

Fig. 1. Circulating myomiRs in ALS patients. Bar graph showing the levels of different myomiRs in the serum of ALS patients before (T0) and after (T1) 6 weeks of physical rehabilitation. We observed a significant down-regulation of miR-1, miR-206, miR-133a, and miR-133b after rehabilitation treatment (**p-value < 0.001; *p-value < 0.05).

Individual expression levels of myomiRNA after rehabilitation are shown in Fig 2. A general decrease of miRNA was observed after training: for miR-206 we observed a strong down-regulation in 13 patients while the data are not detectable for patient number 4. MiR-1 appeared down-regulated in 15 patients. In three patients (pt 13-14-17) there was a slight increment in miR-206 and miR-1. The individual expression levels of miR-133a and miR133b exhibit a similar pattern of deregulation.

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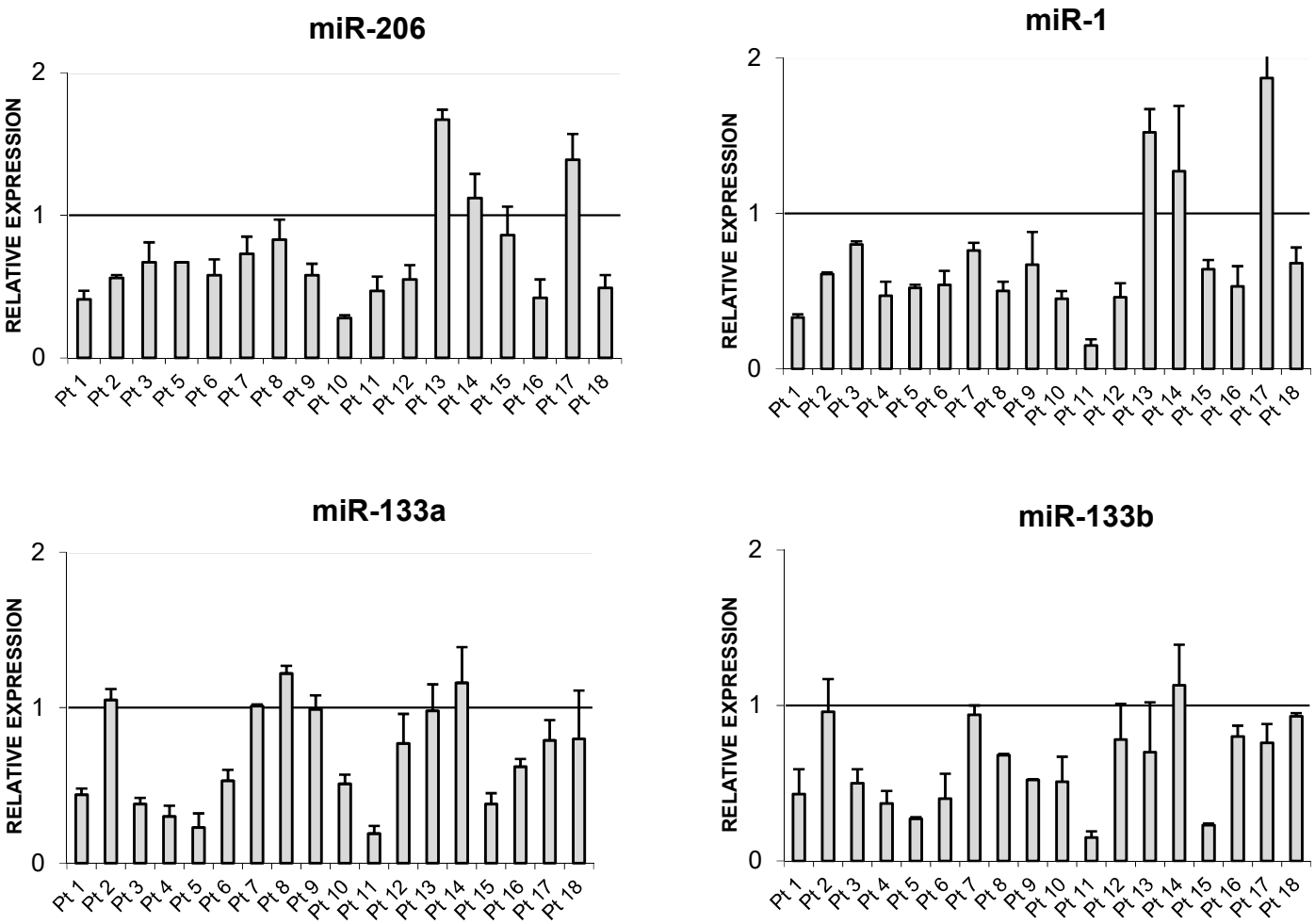

Fig. 2 Expression levels of mir-1, miR-133 a, miR-133b and miR-206 in eighteen ALS patients after 6 weeks of physical rehabilitation. A decreasing trend of miRNA was observed after training showing modulation of myomiRs with rehabilitation.

Discussion

Current medical treatment for ALS is limited to supportive care, and few drugs have been approved for symptomatic treatment. In recent years, the role of skeletal muscle involvement in ALS has been discussed and muscle-specific miRNAs are emerging as biomarkers that are useful for understanding the molecular pathways involved in muscle and NMJ [20]. In this study, we examined the effects of physical rehabilitative practice in patients with ALS, on molecular data on circulating muscle-specific miRNA, the possible biomarkers of muscle regeneration and NMJ recovery and we collected outcome clinical scales before and after training. Physical rehabilitation is standard care for ALS patients admitted to our center (Foundation Hospital San Camillo IRCCS, Venice, Italy) specialized in the rehabilitation of patients with neurodegenerative and neuromuscular diseases.

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The effects of exercise and physical rehabilitation in patients with ALS are under investigation. Moderate and regular exercise is supported in the treatment of numerous muscle conditions [21,22]. Patients with ALS are often advised to avoid exercise to preserve muscle strength and to minimize the effects of possible muscle overload. This recommendation is based on epidemiological studies showing a high incidence of the disease in people who perform an intense physical activity such as, for example, soccer players [23,24]. The role of physical exercise after the onset of the disease has been poorly studied in ALS patients. Evidence in ALS animal models and human studies reinforces the benefits of an exercise program suggesting that moderated endurance exercise can delay disease onset and increase survival. In the SLA mouse model, it was found that physical activity does not affect survival [25]. Moderate and regular physical activity in people affected by ALS shows a temporary positive effect on the symptoms of the disease [26] and improves functional disability scores [27]. In another study, conducted in a few SLA patients with respiratory failure, exercise slowed the clinical deterioration of the disease [28]. Current studies support the effectiveness of exercise in patients with motoneuron disease: two recent clinical trials in ALS patients [9,29] demonstrated safety and tolerability of resistance and endurance training. Lunetta and collaborators [30] proved that monitored exercise programs reduce motor deterioration. Moreover, in healthy people, regular physical activity has many potential physical benefits: reducing pressure, preventing obesity and heart disease, as well as decreasing anxiety, pain [31], and the sense of fatigue.

In accordance with recent reports, we observed a positive effect of physical moderate training: ALS patients had an improvement of muscle strength, physical conditions, and independence documented by a significant change in ALSFR-R, Barthel, and FIM scores. We also investigated the impact of rehabilitation in terms of fatigue, a common symptom in ALS patients [32] that has a negative effect on their quality of life. In our patients we found a decreased sense of fatigue documented by a significant change in FSS index.

The involvement of skeletal muscle in ALS pathogenesis, muscle-specific miRNAs (myomiRs) could be regarded as biomarkers modulating the molecular response to rehabilitation. MiR-1, miR-133a, miR-133b, and miR-206 are designed as canonical myomiRs and they are predominantly expressed in cardiac and skeletal muscle [33]. MyomiRs take part in the molecular network regulating myogenesis, muscle development, repair, and remodeling [34,35]. MiRNA-1 and -206 share specific targets that are closely related to functioning: both promote myoblast differentiation in myotubes and cell proliferation [33,36]. In contrast, the miR-133 family inhibits differentiation by repressing SRF [37].

Mouse models of ALS [38–40] and spinal muscular atrophy [41] have show an up-regulation of miR-206 which is parallel with the onset of the disease and muscle atrophy, and occurs with a disorganization of the NMJ, which might slow down the reinnervation of denervated muscle [38].

High levels of miR-206 are found in ALS; in our previous research we found an increase of myomiRs in ALS patients compared to controls both in serum [42] and in muscle [13].

In the present study, we analyzed the expression levels of muscle-specific microRNAs in the serum of ALS patients before and after a period of six weeks of moderate physical rehabilitation and we observed a significant down-regulation of circulating miR-1, miR-

133a, miR-133b, and miR-206 after training. In ALS, the activation of satellite cells in muscle and NMJ promote tissue regeneration and the reorganization of the muscular fibers to contrast denervation and muscular atrophy [20]. We believe that the decrease in expression levels of myomiRs that we found after training try to restore a normal biological condition in skeletal muscle and NMJ of ALS patients.

The role of miR-206 and miR-133b in the development and maintenance of neuromuscular synapses [43,44] is supported by the fact that they are crucial in ALS disease. Increase of miR-206 and miR-133b has been documented in the muscle of ALS mice models after nerve injury or denervation [38,45,46]. Probably this molecular mechanism is not specific to MND but it is shared between various conditions in which NMJ function is impaired. Several studies support the idea that miR-206 is a key regulator of signaling between muscle fibers and neurons since it acts as a bidirectional sensor of damage or loss of motor neurons and promotes regeneration of functional NMJ to attenuate muscle injury [35,47,48] and delay progression of the disease [38,48]. In addition, the function of miR-133b in NMJ is supported by the observation that this miRNA stimulates neurite growth in the rat brain following nerve damage after treatment with MSC cells [49]. Elevated expression levels of this miRNA found after denervation are related to nerve regeneration.

We found a similar trend of expression in miR-1 and miR-206 and in miR-133a and miR-133b in ALS patients after training that are probably related to common target and functions previously described.

Our data suggest a cross-talk between an upper/lower motor neuron and muscle during rehabilitation. We hypothesize that there is more reinnervation and an increase in synaptic formation documented by miR-206 and miR-133b and less muscle atrophy as an effect of aerobic exercise performance. Our study suggests that myomiRs are correlated in skeletal muscle recovery in response to rehabilitation as we observed a positive effect of moderate rehabilitative training in our sporadic ALS patients documented by the medical scale and a significant reduction of fatigue with a decrement in the FSS scores after exercise. We propose myomiRNA as a valid molecular tool to evaluate the response to rehabilitative treatments in neurodegenerative and neuromuscular diseases.

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