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Unraveling the involvement of muscle-specific microRNAs in motor neuron diseases: evidence from animal models and human patients

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There is freedom waiting for you
On the breezes of the sky
And you ask
"What if I fall?"

Oh but darling,

What if you fly?

Erin Hanson

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ABSTRACT

Motor neuron diseases (MNDs) encompasses a broad spectrum of progressive neurodegenerative conditions characterized by degeneration of upper and/or lower motoneurons. The main MNDs are amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), and spinal and bulbar muscular atrophy (SBMA).
In most cases (90%) ALS occurs in sporadic forms with unknown etiology, whereas the remaining are familial cases, 20% of which account of superoxide dismutase 1 (SOD1) gene mutations. Conversely, SMA and SBMA are exclusively genetic disorders, the former caused by survival motor neuron 1 (SMN1) gene homozygous loss or mutation; the latter is associated with a CAG repeat sequence expansion within exon 1 of the androgen receptor gene.
Despite differences in disease etiology, onset, and progression, MNDs share a common skeletal muscle involvement. Mounting evidence suggests that muscle degeneration might be not only a consequence of motoneuron loss, but myocytes may be the site of crucial pathogenic events, indicating a potential pivotal role of muscle tissue in MND pathogenesis.
MicroRNAs (miRNAs) are key molecules involved in a plethora of physiological and pathophysiological processes and particularly, muscle-specific miRNAs (myomiRs), including miR-1, miR133a, miR-133b, and miR-206, are critical players in myogenic pathways.
The hypothesis of the current project is that myomiRs altered expression in muscle tissue may contribute to MND onset and progression.
The aim of this study was to assess the expression levels of myomiRs and their target genes in muscle tissue of the MND animal models, to investigate their role in the compromised skeletal muscle environment associated to MNDs as therapeutic target and disease biomarkers.
To this purpose, by qPCR we analysed the expression of myomiRs, miR-1, miR-133a, miR-133b e miR-206 and their putative target mRNAs, in G93A-SOD1, Δ7SMA, and AR133Q mouse muscle during disease progression. Target protein levels were assessed by western-blot.
Further, we extended our analysis on serum samples of *SOD1*-ALS, SMA, and SBMA patients, to demonstrate myomiR role as noninvasive biomarkers.
Our data showed a dysregulation of myomiRs and their targets in ALS, SMA, and SBMA mice. In particular, we identified a miR-206 upregulation as a common pathogenic feature associated with MND muscle impairment. A similar myomiR signature was observed in patients' sera.
Our overall findings highlight the role of myomiRs as promising therapeutic molecules and biomarkers in ALS, SMA, and SBMA. Further functional investigations are needed to explore the targeting of myomiR-based drugs to skeletal muscle as novel treatment approaches.

ABSTRACT

Le malattie del motoneurone (MND) raggruppano un ampio spettro di patologie progressive neuromuscolari caratterizzate dalla degenerazione dei motoneuroni superiori e/o inferiori. Le principali malattie del motoneurone sono la sclerosi laterale amiotrofica (SLA), la atrofia muscolare spinale (SMA) e la atrofia muscolare spino-bulbare (SBMA).
L'eziologia della SLA non è completamente nota. Nel 90% dei casi la SLA si presenta in maniera sporadica, mentre nel 10% dei casi è ereditaria, pertanto definita "SLA familiare", dovuta nel 20% dei pazienti a mutazioni nel gene della superossido dismutasi 1 (SOD1). Diversamente, SMA ed SBMA sono malattie monogeniche, la prima dovuta a una perdita in omozigosi o mutazioni del gene di sopravvivenza del motoneurone 1 (SMN1); la seconda è causata da una sequenza espansa CAG nell'esone 1 del gene del recettore degli androgeni.
Nonostante le differenze in termini di eziologici, di manifestazione dei sintomi e di progressione della patologia, le malattie del motoneurone presentano tutte una compromissione a livello muscolare. Numerosi studi suggeriscono che la degenerazione muscolare potrebbe non essere esclusivamente una conseguenza di un danno ai motoneuroni, ma che meccanismi patogenetici avvengano già a livello di cellula muscolare, e che quindi il muscolo possa rivestire un ruolo cruciale nella patogenesi delle malattie del motoneurone.
I microRNA (miRNA), piccoli RNA non codificanti, sono principalmente attivi nella regolazione dell'espressione genica a livello trascrizionale e post-trascrizionale e sono coinvolti in una varietà di processi fisiologici e patologici. In particolare, i microRNA muscolo-specifici (myomiR), tra cui miR-1, miR-133a, miR-133b e miR-1, sono molecole chiave per la miogenesi. L'ipotesi di questo progetto è che l'alterata espressione dei myomiRs nel tessuto muscolare possa contribuire all'insorgenza o progressione delle MND.
Lo scopo del presente studio è stato quello di analizzare i livelli di espressione dei myomiR e dei loro geni target nel tessuto muscolare di modelli animali di malattie del motoneurone, per indagare il loro ruolo nelle alterazioni muscolari associate a tali patologie ed identificare potenziali bersagli terapeutici e biomarcatori.
Mediante real time PCR quantitativa abbiamo analizzato i livelli di espressione dei myomiR quali miR-1, miR-133a, miR-133b e miR-206 e dei loro geni target nel muscolo di topo G93A-SOD1, Δ7SMA e AR133Q durante la progressione della malattia. Mediante western-blot abbiamo anallizzato i livelli proteici dei target. Successivamente abbiamo esteso le analisi a livello di espressione dei miRNA nel siero di pazienti SLA con mutazione della SOD1, SMA ed SBMA.
I nostri risultati hanno evidenziato una alterazione dei livelli dei microRNA muscolo-specifici e dei loto target nei modelli murini di SLA, SMA e SBMA. In particolare abbiamo identificato un aumento significativo dei livelli del miR-206 che risulta essere una caratteristica patogenetica comune alle malattie del motoneurone, associato all'alterazione muscolare. Un trend analogo è stato osservato nel siero dei pazienti.
Complessivamente tali dati suggeriscono che i microRNA possono essere considerati potenziali bersagli terapeutici e biomarcatori per la SLA, la SMA e la SBMA. Studi futuri permetteranno di indagare il potenziale terapeutico dei microRNA muscolo specifici nell'ambito delle malattie del motoneurone.

1. INTRODUCTION

1.1 Motor neuron diseases (MNDs)

Motor neuron diseases (MNDs) are a heterogeneous group of neurodegenerative disorders affecting motor neurons in the motor cortex, brainstem and spinal cord. MNDs affect patients from infancy to adulthood and can either be genetically transmitted or occur sporadically (1).
Geographical heterogeneity in the MND burden might suggest the influence of sociodemographic status and genetic background in different regions. MND burden from 1990 to 2019 has been shown to have globally increased and will continuously spread especially in the middle- and high-income areas. Specifically, the global prevalence and deaths due to MND in 2019 were 1.91% and 12.39% respectively increased compared to 1990. However, since the number of epidemiology studies conducted in South and Central Asia, Latin America, and sub-Saharan Africa is low and MNDs are likely to be underdiagnosed in those areas, the overall actual burden is probably higher than expected. In addition, the ageing of the global population is expected to increase the social burden of neurodegenerative disease, such as ALS that mainly affects the elderly (2). Overall, this evidence highlights the importance of a global healthcare planning.
MNDs are caused by the progressive degeneration of motoneurons causing voluntary muscle weakness gradually worsening over time, leading to severe disability, and often to death (1).
There are two groups of motoneurons, the upper motoneurons (UMNs) and the lower motoneurons (LMNs). The UMN is responsible for integrating all the excitatory and inhibitory signals from the cortex and translating them into a signal that travels down through the axons which synapse directly onto the LMNs in the brainstem motor nuclei and the anterior horn of the spinal cord. The cell body of UMNs is located in the cerebral cortex, and the axon forms the corticobulbar and corticospinal tracts which exert direct (monosynaptic) or indirect (via interneurons) supranuclear control over the LMNs. The LMNs directly innervate skeletal muscles and are responsible for transmitting the signal to the effector muscle to either initiate or inhibit voluntary movements. The muscle fibers are nearly identical, enabling the controlled, synchronous movement of the motor unit, upon depolarization of the LMNs. Lesions of the UMNs cause weakness, hyperreflexia, spasticity, and both positive Babinski and Hoffmann's signs. On the other hand, LMN lesions could occur in the anterior horn of the spinal cord, peripheral nerve, neuromuscular junction, or muscle. This kind of lesions causes hyporeflexia, fasciculations, flaccid paralysis, and muscle atrophy (3).
MNDs exist on a spectrum and the particular clinical classifications and outcomes stem from the specific neuronal component involved in each condition: from a pure lower motoneuron; to mixed upper and lower motoneuron; to a pure upper motoneuron variant in addition to regional variants restricted to the arms, legs or bulbar region. The most common MNDs include: Lou Gehrig's disease, also known as amyotrophic lateral sclerosis (ALS) which presents both upper and lower MN loss; primary lateral sclerosis (PLS) where only UMNs are affected; progressive muscular atrophy (PMA), progressive bulbar palsy (PBP), spinal muscular atrophy (SMA), Kennedy's disease, also known as spinal and bulbar muscular atrophy (SBMA), all characterized by LMN degeneration (4). The present study focused on the three most common MNDs: ALS, SMA and SBMA.

1.1.1 Amyotrophic lateral sclerosis (ALS)

ALS is a neurodegenerative disease that is mainly characterized by the degeneration of UMNs in the cerebral cortex and of LMNs of the brainstem and spinal cord, leading to an initial presentation of the disease that can vary between patients; some present with spinal-onset disease -muscle weakness of the limbs-, whereas others with bulbar-onset disease, which is characterized by dysarthria -difficulty speaking- and dysphagia -difficulty swallowing-. Eventually, the main causes of death are respiratory failure within 3-5 years from diagnosis (5), followed by pneumonia and cardiovascular complications. Nowadays ALS is the most common adult motoneuron disease, with a prevalence of 5 per 100,000 and a lifetime risk of 1:400–1:800 (6). Still, juvenile forms of ALS also exist (jALS) with disease onset occurring typically before the age of 25 and a majority of cases having a decade-long disease progression (7). There are neither clear biomarkers to stratify the patients nor treatments for ALS. To date, the three Food and Drug Administration approved drugs - riluzole, edaravone, and sodium phenylbutyrate-taurursodiol -, only modestly ease the symptoms and slow disease worsening (8).
The phenotypic heterogeneity of ALS has been supported by clinic-based and population-based studies demonstrating that younger age at onset has been found to predict long-term survival of ALS patients, whereas bulbar onset ALS has been invariably identified as a negative prognostic predictor (9).
Nearly all cases of ALS (90%) have neither clearly associated risk factors nor family history, therefore are classified as sporadic ALS (sALS). Although the cause of sALS is largely unknown and there is very little information about the possible exogenous factors involved in ALS, the interplay of environmental factors, genomic and epigenomic 'fingerprinting' could generate the clustering of different phenotypic manifestations (5). On the other side, an estimated 10% of individuals show genetic mutations which run in the family; hence this form is known as familial ALS (fALS). Inheritance of most forms of fALS is autosomal dominant, although autosomal recessive and X-linked dominant transmissions also occur (10). The first discovered ALS-associated gene was superoxide dismutase 1 (SOD1) in 1993 which shows missense mutations in the exon regions, that exerts oxidative stress-related toxic effects as a consequence of protein dysfunction (11). Only in 2006 was a second contributor to ALS described, namely Tar-DNA binding protein (TARDBP), that encodes TDP-43 protein. Pathologic TDP-43 resulted hyper-phosphorylated, ubiquitinated, and cleaved to generate C-terminal fragments (12). In 2009 two independent studies identified mutations in the fused in sarcoma (FUS) (13, 14) which have also been described as the most commonly associated mutations in jALS, linked to the most aggressive form in terms of age of onset and rate of disease progression (7). However, the main discovery dates back to 2011 with the identification of a hexanucleotide $(G4C2)_n$-repeat expansion (HRE) in the non-coding region of chromosome 9 open reading frame 72 (C9ORF72) which accounts for almost 5–10% of patients with sALS and 40–50% of patients with fALS (15). Beyond others ALS-causative genes (*Figure 1*), the four above-mentioned are the most common and responsible for over 10% of sALS cases and 50% of fALS forms (6).

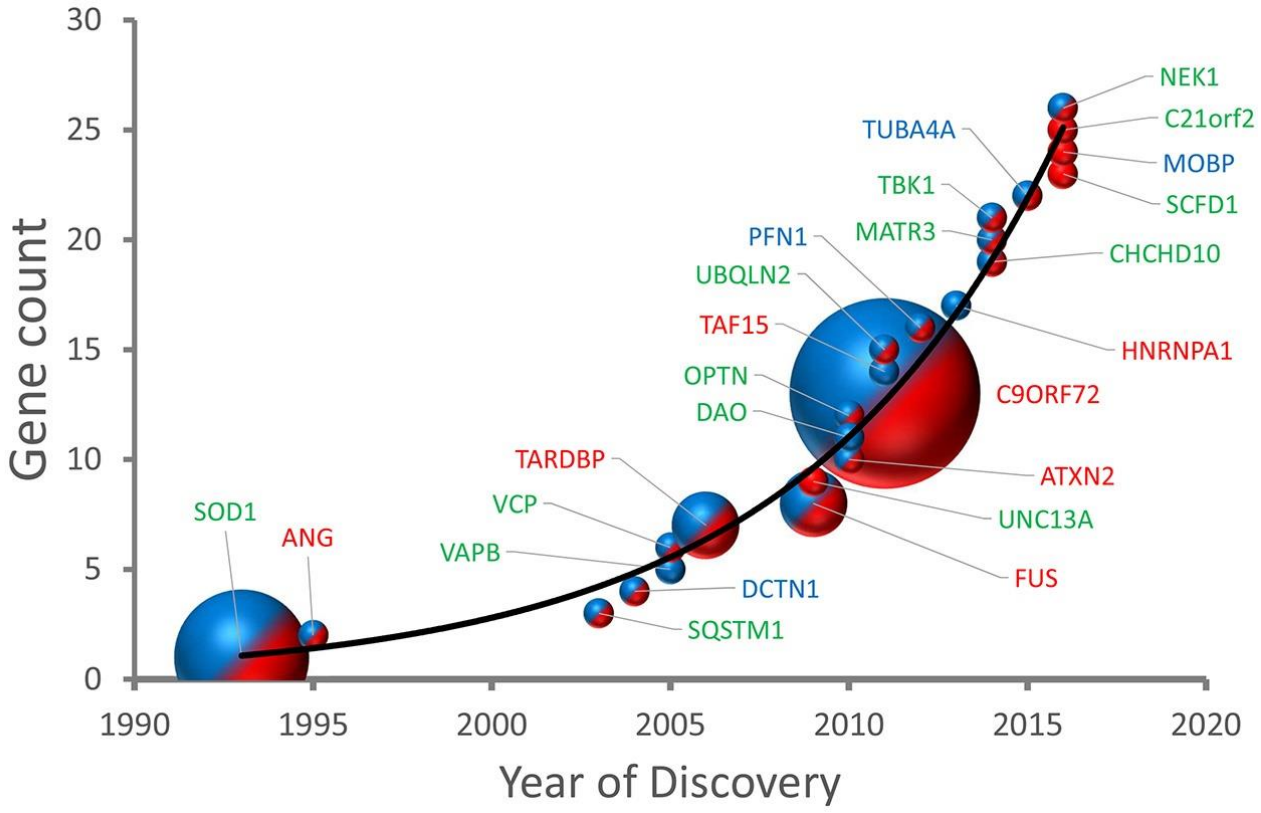


Figure 1. ALS Gene Discovery since 1990. Red circles indicate genes associated only with sALS cases, blue circles indicate genes associated only with fALS cases, and half blue and half red circles indicate genes associated with both sALS and fALS. Genes in green represent those implicated in protein homeostasis, the purple ones represent altered RNA-binding protein and in yellow those representing cytoskeletal proteins. *Adapted from Brown QL et al., 2017*

Most of our current knowledge of the ALS pathogenic mechanisms comes from transgenic mice such as the G93A-SOD1 - carrying a mutant G93A form of the human SOD1 gene in which glycine 93 is changed to alanine - that reliably models the human disease by the development of progressive MN degeneration and clinical signs that closely mimic either the sporadic or familial forms of human ALS. Mice present relatively early onset and rapid disease progression. The clinical phenotype is characterized by an adult onset of motor symptoms in the hind limbs around 12 weeks of age, which progresses to end stage by 17–20 weeks. Pathological signs such as muscle weakness in both upper and lower limbs have been demonstrated by loaded grid test and gait analysis, respectively (16).
Despite intensive research, the poor understanding of disease mechanisms underlying the neurodegeneration/neurotoxicity has hampered the search for effective disease-modifying therapies. However, a generally accepted idea suggests that ALS is most likely the result of a combination of multiple and complex mechanisms, rather than a single pathogenic event, pointing to a multifactorial nature of the disease (17, 18; See *Figure 2*).
Glutamate excitotoxicity was one of the first identified pathogenetic mechanism of ALS. The isoform 2 of the astroglial glutamate transporter (EAAT2) is responsible for keeping the amount of glutamate below excitotoxic level in the nervous system. It has been found that the motor cortex and spinal cord of ALS patients and transgenic mutant SOD1 mouse models presented reduced EAAT2 levels leading to synaptic glutamate concentration increase. This generically triggers excitotoxic neuronal degeneration, by sustained influx of intracellular calcium levels and formation of free radicals or reactive oxygen species (ROS) (17).
Crucial mitochondrial damages have been related to many neurodegenerative diseases including ALS. The pathogenic cascade results in abnormal production of ATP and ROS, dysfunction in energy

and calcium homeostasis, eventually leading to apoptosis. Moreover, altered protein expression and mitochondrial transport along axons have been reported in both ALS transgenic mice and patients (17). Finally, a key role is played by impaired clearance of damaged mitochondria by autophagy. Defects in the autophagic pathway have also been linked to another pathogenic mechanism occurring in ALS, the impaired proteostasis. In particular, there is evidence that disruption of the two main protein clearance pathways - autophagy and the Ubiquitin-proteasome system (UPS) - leads to the formation of misfolded proteins, which oligomerize and aggregate, gaining toxic properties. Interestingly, up to 80% of ALS cases presents TDP43 aberrant protein inclusions, but protein aggregates of the other most commonly mutated ALS genes (SOD1, C9ORF72, and FUS) all give rise to proteins that are found to aggregate in several districts in ALS patients (19).

Recently, studies suggest a direct role between DNA damage and ALS. Evidence of DNA damage accumulation and DNA repair deficiency is emerging, highlighting the role of genomic instability as hallmark of disease pathogenesis.

Lastly, a worth mentioning pathogenic event linked to ALS is neuroinflammation, that has been described in many other neurodegenerative diseases. ALS neuroinflammation is characterized by activated microglia, astrogliosis and infiltrating immune cells in the sites of neuronal injury. Interestingly, it was one of the first pathogenic mechanisms - since the early 2000s – suggesting the key role of non-neuronal cell types in triggering or supporting the ALS neuromuscular degenerative processes, a theory called “non-cell autonomous mechanism”, meaning that a deleterious interplay between different cell types is necessary to trigger the pathogenesis of the disease (20). Indeed, for decades, ALS has been considered a “neurocentric” disease. This longstanding neurocentric view of ALS stems from the idea that MN degeneration in ALS is a cell-autonomous process independent of external influences, and that muscle alterations in the periphery only represent a consequence of the MN loss (21, 22). However, mounting evidence is gaining insights into the role of nonneuronal neighboring cells in acting mutually and synergistically to exacerbate MN degeneration in ALS, broadening our knowledge about the pathogenesis of the disease.

Astrocytes are clearly affected in ALS: in the transgenic SOD1 mouse early Lewy-body-like inclusions are present, prior to glial activation and prior to the accumulation of inclusions in neurons. Moreover, astrocytes in ALS patient tissues show cytoplasmic TDP43 inclusions, and the overexpression of mutant TDP43 in astrocytes alone is sufficient to trigger MN death in animals.

Microglia become activated early in ALS, at or before disease onset in mutant SOD1 mice and with the number of cells increasing with progression. It has been suggested that microglia induce MN death via the NF-κB pathway, that is highly activated in microglia since disease onset in the SOD1 mouse (23).

There is also significant evidence that oligodendrocytes contribute to ALS pathologies in mouse models. Removal of mutant SOD1, specifically from oligodendrocytes, extends survival of SOD1 mice (24).

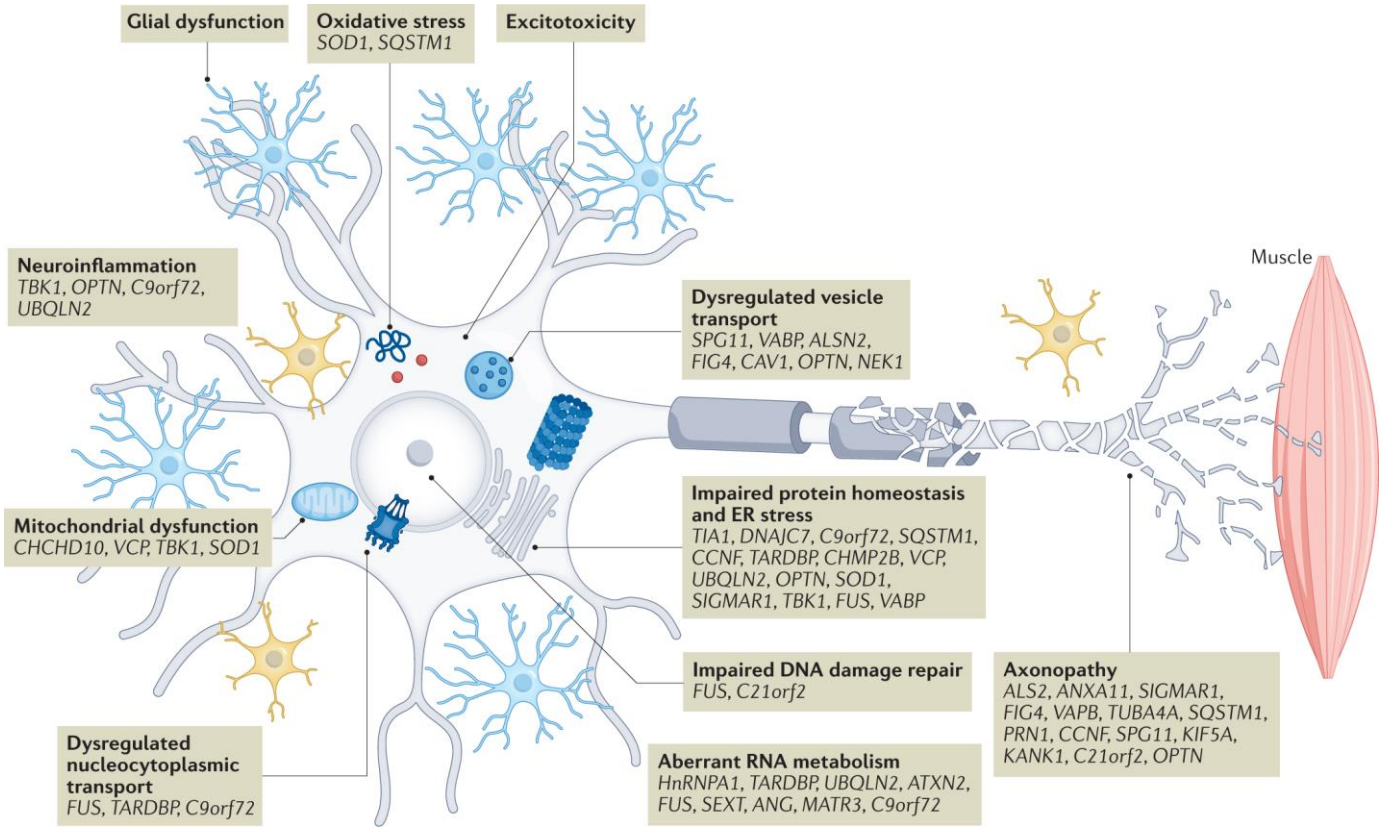


Figure 2. ALS pathophysiology. The main pathological processes leading to ALS are presented in light brown boxes, along with related genes. *Adapted from Mead RJ et al., 2022.*

1.1.1.1 Skeletal muscle in the ALS pathology

Muscle atrophy plays a crucial role in MN diseases, and contributes to ALS onset and progression (25, 26). It is undefined whether MN degeneration in ALS is triggered intrinsically, and hence is autonomous, or if the disease-initiating mechanisms are extrinsic to MNs and highly influenced by muscle atrophy (26).

However, the exact sequence of pathogenic changes resulting in ALS is not completely clear. Two hypotheses have been delineated: the 'dying forward' and the 'dying back' theories (27). According to the 'dying forward' hypothesis, MN dysfunctions, appearing as hyperexcitability, start within the cell body of cerebral cortex MNs (i.e. upper MNs) (28, 29); subsequently, the brainstem and spinal cord MNs, connecting upper MNs to muscle (i.e. lower MNs), are affected by a glutamatergic excitotoxicity that modifies synaptic transmission at NMJs (28).

By contrast, the 'dying back' hypothesis postulates that MN dysfunctions begin distally in the periphery, specifically in the lower MN axons, and progress towards the lower MN cell body (30-32). Based on this theory, ALS is a distal axonopathy, whereby the disease originates from NMJs and skeletal muscle, then leads to lower and upper MN death via a retrograde signaling cascade (26, 33). Therefore, our previous study demonstrates that muscle atrophy occurs early in the life of G93A-SOD1 mice, before any evidence of neurodegeneration (34), supporting the idea that ALS can initiate from muscle damage. In this line, empirical and clinical evidence demonstrated that restricted expression of mSOD1 to MN did not trigger the pathology (35), but mSOD1 expression in skeletal muscle elicited muscle atrophy by localized oxidative stress, decreased muscle strength, impaired mitochondrial distribution and the contractile apparatus, reduced the spinal cord mass, triggered late MN loss, and shortened mouse lifespan (26, 30). Furthermore, Fischer and colleagues found skeletal

muscle denervation and reinnervation abnormalities in human ALS biopsies when MN degeneration was not yet evident (36). By in vitro experiments, muscle satellite cell abnormalities (37) and decreased capacity to generate mature myotubes was observed in myoblasts derived from ALS patients, suggesting a crucial role of muscle in the disease onset and progression (38, 39). Based on the 'dying back' hypothesis, where the MN survival could be influenced by trophic factors released from muscle, preclinical therapeutic approaches targeting and modulating skeletal muscle functions have been developed (40). For instance, the administration of trophic factors, such insulin-like growth factor 1 (IGF1) and growth hormone (GH), are able to stimulate myogenesis by modulate of protein synthesis. The upregulation of IGF1 in muscle ameliorate hindlimb muscle strength and MN survival. It was described that muscle-restricted expression of IGF-1 isoform preserved muscle architecture and induce the satellite cell activity in G93A-SOD1 transgenic mice, stimulating the calcineurin-mediated regenerative pathways (41). In addition, pre-clinical studies found that the glial cell-derived neurotrophic factor (GDNF), ameliorates MN sprouting ability and survival (42), whereas since vascular endothelial growth factor (VEGF) promotes angiogenesis and neuronal survival (43), vascular endothelial growth factor (VEGF) supplementation in skeletal muscle of SOD1 mice had positive effects on ALS symptoms (44). Moreover, metabolic modulators have been tested, like trimetazidine, which acts on mitochondrial protein levels and energy metabolism, stimulates myogenesis and oxidative metabolism in muscles, improving NMJ and neuromuscular communication and ultimately extending survival of SOD1 mice (45). Other approaches aimed at targeting alterations in ion channel function, such as acetazolamide, which restores ClC-1 chloride channel activity and sarcolemma hyperexcitability have been studied in human skeletal muscle (46). Lastly, physical activity can promote myofiber regeneration by activating satellite cells with improvements on metabolism and mitochondrial biogenesis. In particular, swim training affects Akt signaling and ameliorates loss of skeletal muscle mass in the ALS mouse, showing a beneficial effect on MN survival (47).

Altogether, these studies provide unclouded evidence of the crucial involvement of skeletal muscle in ALS pathogenesis and challenge the well-established dogma that muscle atrophy merely occurs as a consequence of MN degeneration. Although the concrete ways through which muscle pathology can affect MNs post-synaptically remain unanswered, the understanding and identification of the initial mechanisms leading to the progressive loss of motoneurons in ALS is crucial to identify a target tissue to address novel therapeutic treatments.

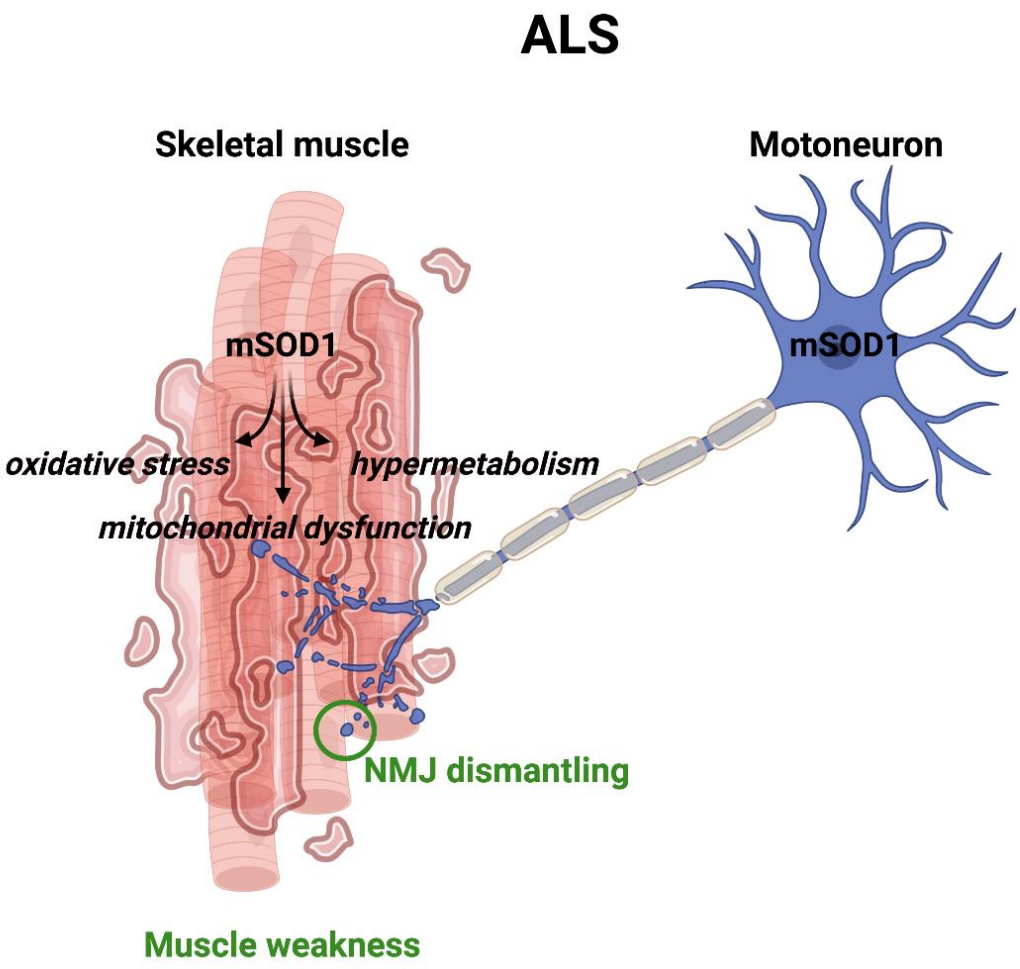


Figure 3. Potential pathogenic mechanisms in SOD1-linked ALS skeletal muscle. Mutant SOD1 expression in skeletal muscle leads to oxidative stress and mitochondrial dysfunction, resulting in NMJ dismantling and muscle atrophy and weakness (green). mSOD1 mice skeletal muscles are also hypermetabolic but whether mSOD1 expression in muscle or in other cell types is responsible of this phenotype is unknown. Muscle hypermetabolism contribute to drive NMJ destruction and systemic energy deficit. Created with BioRender.com

1.1.2 Spinal muscular atrophy (SMA)

SMA is a neuromuscular autosomal recessive disorder occurring in approximately 1 in 10,000 live births and representing the second most common fetal autosomal disorder after fibrosis cystic (48, 49). It is characterized by the degeneration of the anterior horn cells of the spinal cord and motor nuclei in the lower brainstem, leading to a spectrum of phenotypes but, in general, patients with *SMA* experience a progressive loss of muscle strength and control. More than 95% of the SMA cases are linked to homozygous mutations in the survival motoneuron (SMN) gene located on chromosome 5q13 (designated as 5q-SMA). Interestingly, an expanding and complex group in the 5% of cases consists of rare spinal muscular atrophies (non-5q SMA), which are genetically and clinically heterogeneous and result from genetic abnormalities in genes other than SMN (50). SMN exists in two almost identical variants, the telomeric SMN1 and the centromeric SMN2. SMN2 differs in the regulation of exon splicing, since a C>T change results in exon skipping which generates a truncated isoform of the SMN protein, less stable compared to the full-length produced by SMN1 and often rapidly degraded (51). In the 95% of the 5q-SMA cases the disease is due to a homozygous deletion in SMN1, whereas the remaining 5% of patients presents a heterozygous deletion of SMN1. The

differences in SMN protein activity are inversely correlated with the number of SMN2 copies that can partially compensate for SMN1 loss. Therefore, the greater the SMN2 copies, the later the onset of disease symptoms and the milder the disease course. Indeed, the complete absence of any form of SMN is embryonically lethal (52), suggesting that SMA is a prenatal rather than a postnatal disease. Notably, several factors have been suggested as SMA severity modifiers, such as Plastin3, NAIP, H4N4, IGF1, ZPR1 and UBA1, which are capable of rescuing defects associated with SMN loss. However, they cannot fully compensate for SMN functions (53).

SMA is classified in type 1 (Werdnig-Hoffmann disease), type 2 (intermediate or Dubowitz syndrome), type 3 (Kugelberg-Welander disease), and type 4 (adult type). SMA 1 is the most common and aggressive form, representing 45% of SMA cases and is associated with onset before age 6 months, while death occurs within 2 years. Infants with type 1 SMA usually have 2 or 3 copies of SMN2. An uncommon and unusually severe clinical phenotype has been recognized in some infants, and the classification of type 0 SMA is sometimes used. Respiratory insufficiency is present at birth, and death typically occurs within weeks of birth.

Type 2 SMA represents the 20% of cases and is usually associated with 3 SMN2 copies. The majority of patients with type 2 SMA survive to age 25 with a severe lower limb weakness.

The 30% of SMA patients are affected by type 3 SMA, having 3–4 copies of SMN2; disease onset appears between age 18 and 30 but there are no consequences on life expectancy. Very similar in terms of symptoms is type 4 SMA, representing less than 5% of total SMA's, affecting patients who usually have 4 copies or more, and therefore it is the mildest form of SMA. The presentation is distinguished solely on later onset in adulthood (51).

SMA is a multistemic disease with cellular and molecular changes in skeletal muscle, heart, kidney, liver, pancreas, spleen, bone, connective tissues, and immune systems (54, 55). Treatment consists of managing the symptoms and preventing complications. There is growing evidence, based on the first nusinersen-treated patient's long-term follow up, that the timing of drug intervention is crucial regardless of disease severity (56). Nusinersen/Spinraza™ is the first FDA approved drug for pediatric and adult SMA patients. This antisense oligonucleotide drug regulates the pre-mRNA splicing of the SMN2 protein. However, because of its intrathecally administration, the effects are limited to the central nervous system (CNS) MNs and do not affect SMN levels in other systemic tissues. The one-time dose Onasemnogene abeparvovec/Zolgensma™, approved in 2019, uses an adeno-associated virus 9 (AAV9)-based gene replacement therapy approach to directly provide SMN1 gene in order to produce the full length SMN protein. The main advantage of this approach is that a one-time injection is sufficient and leads to the systemic expression of the SMN protein. However, its long-term transgene expression is expected to be limited to post-mitotic cells such as neurons. Patients under 2 years of age can benefit from Onasemnogene abeparvovec therapy or the small molecule Risdiplam/Evrysdi™ can also be used. Risdiplam is an oral formulation approved in 2020 that acts as an SMN2 gene splicing modifier, leading to higher levels of SMN protein. Moreover, being an oral administration, a systemic tissue delivery is also ensured (57). Conversely, targeting the splicing machinery, it may also affect other transcripts, leading to unknown off-target side effects.

Mouse models of SMA offer a rich source to examine multi-organ effects of low SMN and test various therapeutic strategies for SMA. In particular, Δ7SMA mice exhibit a systemic reduction of the SMN protein (58).

Since its discovery as a SMA-determining gene in 1995 (59), SMN has been widely studied. It is ubiquitously expressed and found both in cytoplasm and nucleus. Due to its systemic loss, many studies have suggested that different organs, including skeletal muscle, heart, and liver, contribute to the SMA phenotype (58). SMN canonical function is related to the small nuclear ribonucleoprotein (snRNP) assembly (59, 60); indeed, it has been shown that SMN deficiency causes widespread defects in splicing (53) in a tissue-specific fashion (61). To date, it is clear that SMN plays extensively other roles, such as in small nucleolar ribonucleoproteins (snoRNPs), which are associated with the post-transcriptional processing and modification of ribosomal RNA in the nucleolus. Moreover, it is also involved in telomerase biogenesis, RNA processing such as transport and local translation,

important neuronal functions such as cytoskeletal dynamics and endocytosis, protein turnover processes of autophagy and ubiquitin–proteasome pathway, and regulation of mitochondrial activity (ensuring specificity of assembly and correct trafficking) (61).

1.1.2.1 Skeletal muscle in the SMA pathology

The muscle-specific role of SMN is rather less clear, although restricted depletion of SMN exon 7 in mouse skeletal muscle is known to cause severe muscular dystrophy and weaken regenerating processes, interestingly, without promoting MN degeneration (62). Moreover, skeletal muscle of SMN mice and in vitro data on *SMN* knockdown C2C12 myoblasts demonstrated a decreased myoblast proliferation and impaired myotube fusion, as already observed in type 1 SMA patient's primary muscle cells (58, 63). SMN function in skeletal muscle tissue is likely to be related to its sarcomeric structure, being the SMN complex localized at sarcomeric z-discs in striated myofibrils (64), and acetylcholine receptor (AChR) formation. Indeed, NMJs of SMA mice showed impaired maturation of the AChR clusters reflected in functional deficits at the NMJ characterized by intermittent neurotransmission failures (65).
Altogether, it has been proposed that SMA could be considered as a developmental NMJ synaptopathy, underlying the key role for *SMN* in muscle homeostasis and development (65).
Given the genetic etiology of SMA, the restoration of SMN expression in skeletal muscle should not be overlooked when developing therapeutic strategies. However, supplemental interventions could support the muscle atrophy phenotype of SMA. In this line, genetic modulation of the myostatin/follistatin axis in skeletal muscle has been tested in SMA animal model. The delivery of the recombinant follistatin increased muscle mass and body weight, improved motor function and also increased MN numbers and size in SMA model mice. Moreover, the genetic muscle overexpression of IGF-1 onto the same SMA background used in the follistatin studies led to increased muscle mass as well as prolonged mouse lifespan (66). Lastly, an oral formulation acting as a troponin activator, Reldesemtiv, that sensitizes the sarcomere response to calcium by modulating the troponin complex, is currently being studied in a wider cohort of patients to test whether this treatment also yields positive results in terms of motor functions. Although these studies are in progress the obtained data are interesting and promising (67, 68).

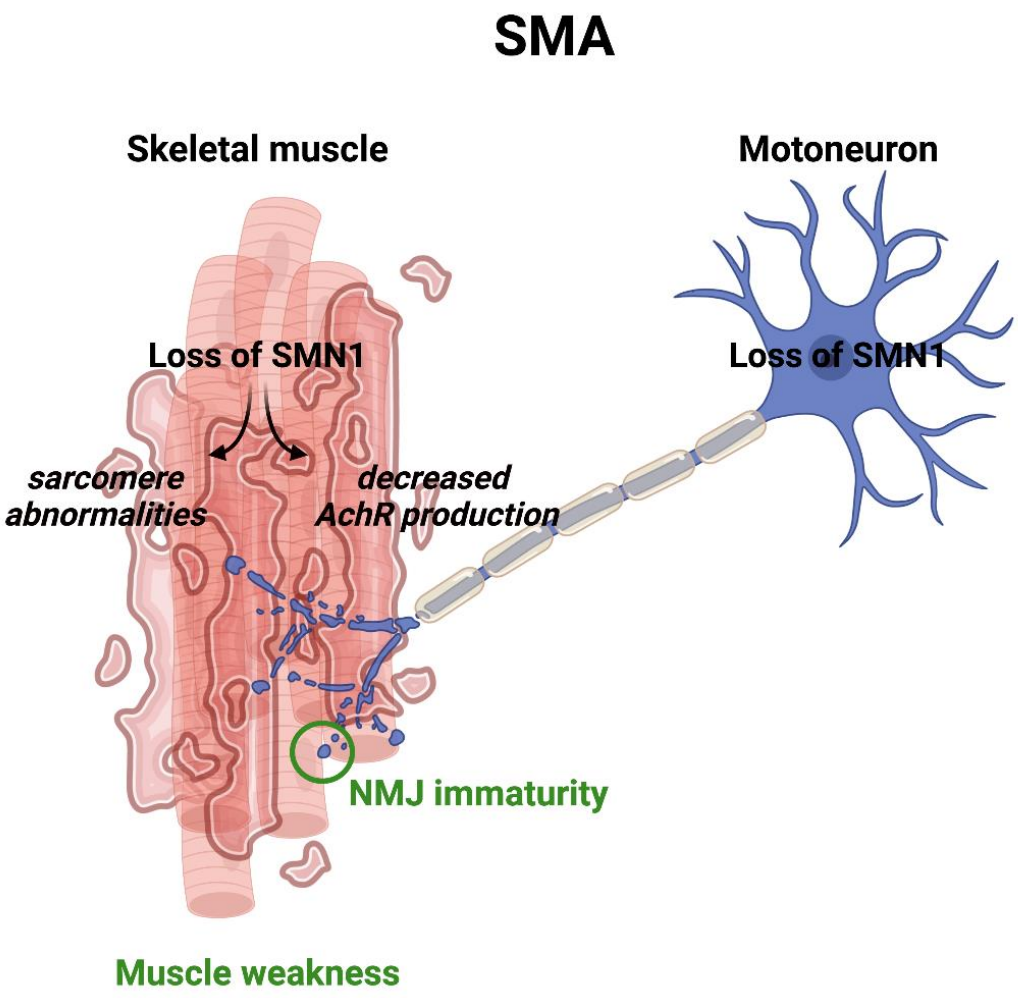


Figure 4. Potential pathogenic mechanisms in SMA skeletal muscle. Loss of SMN1 leads to first, abnormalities in sarcomere structure, which are a likely cause of muscle weakness (green). Second, decreased potential of muscle to produce mature AchR subunits, leading to an abnormal development of NMJs (green). Created with BioRender.com

1.1.3 Spinal and bulbar muscular atrophy (SBMA)

SBMA is an adult-onset X-linked recessive neurodegenerative disorder related to abnormal pathological CAG trinucleotide/glutamine repeat expansion within the exon 1 of the androgen receptor (AR) gene (located on the chromosome Xq12), one of the 16 known disease genes associated with non5q-*SMA* (69), leading to the formation of an expanded polyglutamine (polyQ) tract in the AR protein (70).In normal individuals, the polyQ tract length ranges from 5 to 36 repeats, whereas expansions over 38 repeats cause disease (71).
The prevalence is estimated to be around 1–2 per 100,000, however, the exact data is still unknown, since the disease is thought to be underdiagnosed (72, 73). There is a well-established phenomenon of inverted correlation between age of onset and the length of the CAG repeat expansion with marked earlier age at onset in patients with longer repeats. Being an X-linked pathology, the disease is prevalent in men. However, females carrying the homozygous mutation can experience a mild form of SBMA. Symptoms start in adulthood with hand tremors and muscle twitching. As the condition progresses, limb weakness and motor deficits occur. Due to the involvement of the bulbar region, dysfunctions in face and tongue muscle result in difficulty speech and swallowing. Additionally, some patients experience androgen insensitivity with the development of enlarged breasts (gynecomastia)

or reduced fertility due to testicular atrophy or shrinking. The condition begins late in life and progresses relatively slowly, therefore earlier diagnosis and supportive care measures can prevent or delay disease worsening (74).
A knock-in mouse model for SBMA was made by replacing 1,340 bp of the coding sequence of mouse AR exon 1 with hAR exon 1 sequence containing 113 CAGs. In the transgenic AR113Q mice, the polyQ AR was able to effectively masculinize the male mice, likely because the AR113Q is under the regulatory control of the mouse AR gene promoter. The AR113Q knock-in mice show signs of motor deficits and partial androgen insensitivity, including testicular atrophy and decreased fertility, which are seen in SBMA patients. A striking age-dependent testicular pathology was observed in the AR113Q. These results lead to the conclusion that the abnormalities in testicular morphology in AR113Q were mediated not only by a partial loss of AR function, but also reflect a "toxic" gain-of-function due to AR polyglutamine tract expansion (74). At the molecular level the mutant receptor has normal ligand biding, and in the presence of either testosterone or dihydrotestosterone it dissociates from cytoplasmic heat shock proteins (HSPs) and is normally taken up into the nucleus. Although the mutation leads to a partial loss of transactivation function and subsequent androgen insensitivity, SBMA is predominantly associated with toxic gain of function properties of the polyQ-expanded AR, such as triggering androgen-dependent protein unfolding and mutant AR intranuclear translocation and aggregation, which ultimately result in loss of lower motoneurons in the brainstem and spinal cord of patients (71, 75). Mutant AR aggregates are insoluble, and it has been shown that they interact and sequester other molecules such as caspases - pointing to an involvement of apoptosis in this pathogenic mechanism - and ubiquitin proteasome system (UPS) components and chaperonins, suggesting a role of proteolysis disruption in the disease (75). First detected in motoneurons, intranuclear aggregates have also been identified in motoneurons, NMJ, and skeletal muscle, where AR is especially required for the maintenance in muscle mass and fiber type as well as muscle strength in males (65). In addition, the polyglutamine-expanded AR induces diverse molecular events, such as transcriptional and post-translational dysregulations, axonal transport disruption, loss of neurotrophic support and mitochondrial dysfunction (74).

1.1.3.1 Skeletal muscle in the SBMA pathology

The idea that SBMA originates from neuronal degeneration has been broadened to include the evidence that the disorder may also have a myogenic contribution. Indeed, the selective overexpression of normal AR in rat skeletal muscle, but not motoneurons, resulted in an androgen-dependent SBMA-resembling phenotype, indicating that dysregulation of androgen signaling in muscle tissue is critical for the maintenance of muscle homeostasis. Moreover, in SBMA patients and early in the AR113Q transgenic mice, a progressive glycolytic-to-oxidative fiber type switch was observed, that may reflect a selective sensitivity of glycolytic skeletal muscle fibers to polyQ-AR toxicity, prior to any neurodegeneration (72, 76). Interestingly, the AR113Q knock-in mice developed early androgen-dependent neuromuscular weakness, and myopathic and neurogenic skeletal muscle pathology was observed before neuronal intranuclear inclusions were detected in the spinal cord. Moreover, ASO-dependent polyQ AR suppression in skeletal muscle of two SBMA mouse models is sufficient to ameliorate disease phenotype (77). SBMA patients often exhibit features of myopathy and myogenic abnormalities in muscle tissue, including atrophic and morphologically abnormal angulated muscle fibers, fiber-type grouping, necrosis and macrophage infiltration, and centralized nuclei, all features associated with primary myogenic changes (66, 72). In addition, toxic effects of polyQ-AR in skeletal muscle interfere with IL-4 expression during SBMA patient myotube fusion in vitro (78). Overall these findings raised the prospect that diseases such as SBMA that have been regarded as "motoneuron diseases" result from processes that originate in muscle, and eventually cause pathology in motoneurons (74).

Considering this evidence, it is not surprising that the best-characterized metabolic indicator of SBMA so far is serum creatine kinase (CK), which is elevated to approximately 3 to 4 times the normal range in the majority of SBMA patients. This is higher than would be expected for a purely neurogenic disease, underscoring the fact that primary myopathy contributes to SBMA (79, 80).

So far, not many skeletal muscle-targeting strategies have been developed and tested in the context of SBMA. It should not be forgotten that skeletal muscle provides trophic support for motoneuron innervation, via expression of key growth factors and electrical impulses that drive motoneuron function and, importantly, reduced expression of key neurotrophic factors - including VEGF, type II transforming growth factor-beta receptor β (TGF- β), GDNF and neurotrophin-4 (NT-4) - has been demonstrated in SBMA. Thus, polyQ-AR mediated transcriptional dysregulation of growth factors and neuromuscular electrical activity could directly diminish the trophic ability of SBMA skeletal muscle. In support of this hypothesis, genes implicated in muscle function, myogenesis, and energy balance are dysregulated in the skeletal muscle of SBMA models. Therapeutic approaches could either involve removal of polyQ-AR in muscle, thus restoring its ability to sustain and nourish motoneurons (76) or provide muscle with growth factors in the attempt to counteract MN dying back. Indeed, skeletal muscle overexpression of Igf-1, that acts upstream of Akt, reduced AR nuclear aggregates, delayed disease onset and extended survival in SBMA model mice, improving motor behavior and reducing motoneuron cell loss. Igf-1 has previously been shown to benefit motoneurons by promoting sprouting and axonal growth, therefore Igf-1 may improve motoneuron health via its trophic properties in addition to its effects on mutant AR. These data therefore suggest that a pharmacological intervention solely targeted at the muscles is able to provide global protection to motoneurons (81).

Lastly, as previously mentioned, exercise improves function in both myopathies and motoneuron diseases, suggesting that exercise could be beneficial also for SBMA patients. Furthermore, exercise has been shown to increase levels of Igf-1, stimulating proliferation of satellite cells. However, conventional aerobic training had no clear effects on motor capabilities in SBMA patients and may even promote fatigue, but alternative training forms might be tested, since encouraging preliminary data have demonstrated that fitness improves with high-intensity aerobic training (82).

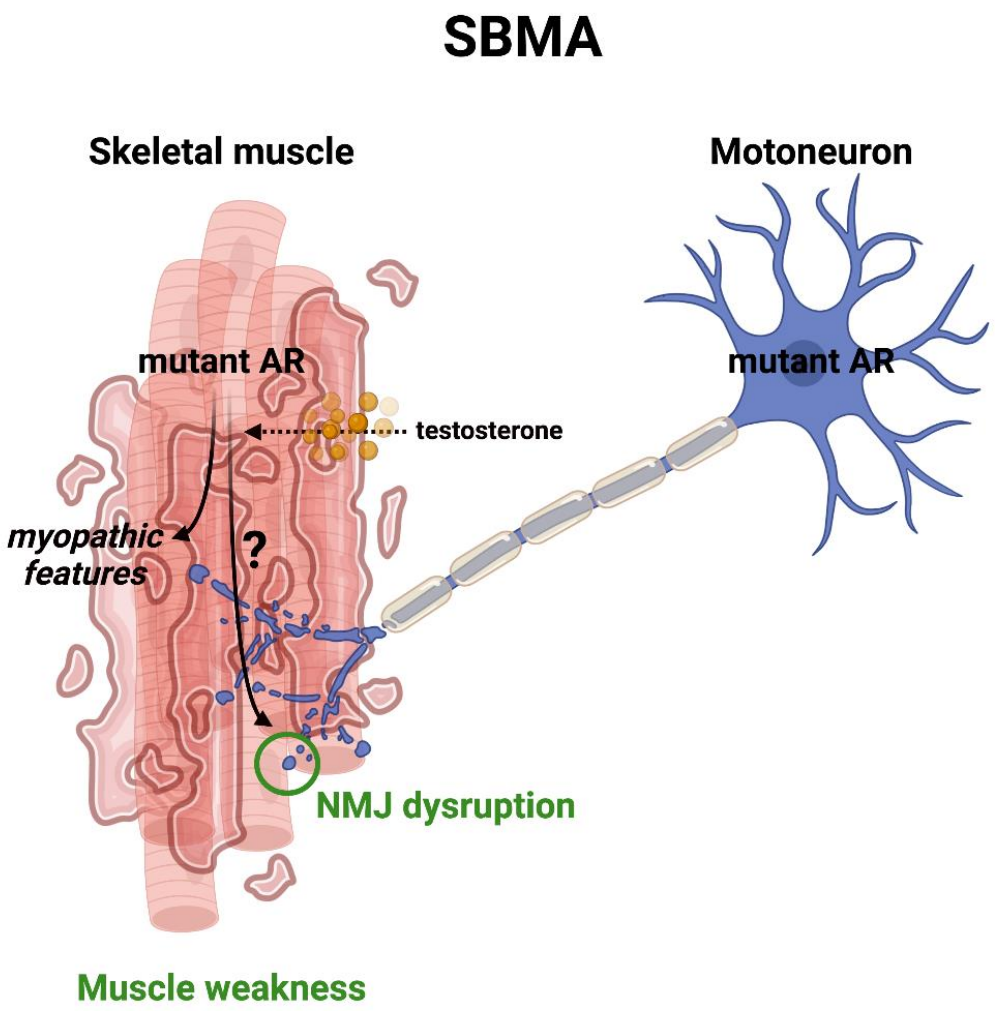


Figure 5. Potential pathogenic mechanisms in SBMA skeletal muscle. The mutant AR toxicity is unmasked by testosterone. This leads to myopathic features and increase of blood CK, contributing to muscle weakness (green). On the other hand, mutant AR toxicity (or wild type AR overexpression) dismantles NMJs (green) through yet unknown mechanisms. Created with BioRender.com

1.2 MicroRNAs (MiRNAs)

98–99% of the human genome, consisting of non-coding DNA, was dismissively defined by Susumu Ohno in 1972 as "junk DNA" (83), as it appeared to have accumulated over time without having any biological purpose. The discovery and validation of microRNAs (miRNAs) in the nematode *Caenorhabditis elegans* by Lee and colleagues in 1993 refute once for all the erroneous "junk DNA" hypothesis, describing them as tiny regulators with great potential. Indeed, with an average of 22 nucleotides in length (84) these single strand molecules are able to switch off or fine-tune gene expression in a great variety of physiological and pathological processes, through post-transcriptional and transcriptional gene-modulating mechanisms (85) accounting for the greater complexity of higher eukaryotes. It is remarkable that these tiny RNA molecules with such important and ubiquitous roles in regulating cellular pathways evaded the scientific community's radar for most of the 20th century (86). Since their discovery, intense research aimed at finding new members of this family. As a result, a multitude of miRNAs was identified among different species of plants and animals, all collected for the first time in 2002 in an online repository called miRbase, where potential miRNA sequences, nomenclature, and target prediction information were listed (87).

1.2.1 MiRNA biogenesis

There are many roads to miRNA maturity (*Figure 6*). Based on their location in the genome, miRNA genes are generally classified as "intergenic" -located between genes-, or "intragenic" -processed mostly from introns, but in few cases from exons, of protein-coding transcripts-. The first step of miRNA biogenesis consists in the transcription of miRNA genes into primary miRNA transcripts (pri-miRNA). While intergenic miRNAs are transcribed from their specific promoters into pri-miRNAs, the pri-miRNAs of intronic miRNAs are considered as the heterogeneous nuclear RNA (hnRNA) of their respective host genes, but the subsequent steps of biogenesis are the same as for intergenic miRNAs (85).

Either RNA polymerase II or RNA polymerase III are responsible for the hairpin-like pri-miRNA generation. Many pri-miRNAs polyadenylated by a 3' poly(A) tail and capped with 5' 7-methylguanosine (m^7G) are transcribed by polymerase II, whereas miRNAs encoded by the largest human miRNA cluster, named C19MC, are transcribed by polymerase III. An average human pri-miRNA contains a hairpin stem of 33 base-pairs, a terminal loop and two single-stranded flanking regions upstream and downstream of the hairpin. The pri-miRNA is then endonucleolytically cleaved by the nuclear microprocessor complex formed by the DiGeorge critical region 8 (DGCR8) protein and the RNase III enzyme Drosha.

DGCR8 contains two double-stranded RNA-binding domains, essential for miRNA processing in all tested organisms. DGCR8 directly and stably interacts with the pri-miRNA and functions as a molecular ruler to determine the precise cleavage site. The two RNase domains of Drosha cleave the 5′ and 3′ arms of the pri-miRNA hairpin, exciding 11 base pairs away from the single-stranded RNA/double-stranded RNA junction at the base of the hairpin stem, and producing the ~70- to 120-nucleotide-long pre-miRNA with a 5′ phosphate and ~2-nucleotide 3′ overhang. A single nucleotide polymorphism in a miRNA precursor stem can block Drosha processing. However, many miRNA sequence aberrations observed in human tumors alter the secondary structure without affecting processing and reveal the structural flexibility of the microprocessor.

Drosha-mediated cleavage can also be regulated for individual miRNAs: about 14% of the human pri-miRNA loops are conserved between different species and could provide anchor points for similar regulatory mechanisms. Moreover, the two components of the microprocessor complex regulate each other: DGCR8 stabilizes Drosha, whereas Dgcr8 mRNA is degraded by DROSHA through a negative feedback loop reducing Dgcr8 expression when sufficient microprocessor activity is available.

Surprisingly, a group of intron-derived miRNAs called mirtrons, discovered in several species, including mammals, are able to bypass Drosha-mediated processing into pre-miRNAs if properly sized to form a hairpin resembling a pre-miRNA. Subsequently, they are released from their host transcripts and further processed in the cytoplasm.

After nuclear processing, the pre-miRNA is exported into the cytoplasm by the karyopherin Exportin-5 (XPO5) in complex with a Ran-dependent nuclear transport receptor protein (Ran-GTP). XPO5 recognizes the pre-miRNA independently of its sequence or the loop structure. A defined length of the double-stranded stem and the 3′ overhangs are important for successful binding to XPO5, ensuring the export of only correctly processed pre-miRNAs.

Further cytoplasmic pre-miRNA processing is mediated by RISC complex, whose assembly is mediated by the RISC loading complex (RLC). RLC is a multi-protein complex composed of the RNase type III Dicer, the double-stranded RNA-binding domain proteins TRBP (Tar RNA binding protein) and PACT (protein activator of PKR), and the core component Argonaute-2 (Ago2). TRBP and PACT facilitate and stabilize Dicer-mediated cleavage of the pre-miRNA (88, 89).

In the cytoplasm the pre-miRNA undergoes an endoribonucleolytic reaction via Dicer, resulting in a mature miRNA duplex with removal of the terminal loop and a 3′ overhang at both ends. One strand is selected as the miRNA effector or "guide strand" based on various factors such as the thermodynamic stability of the 5′ end of the duplex and is loaded onto the Ago2 in an ATP-dependent

manner (90). The "passenger strand (or miR*)" is degraded or, rarely, even associates with AGO proteins, enabling both strands to serve as functional miRNAs (86).
The guide strand is led to the complementary sequence at 3′ UTR of the target messenger RNA (mRNA), called miRNA response elements (MREs), to pair with its 5′-proximal seed sequence (nucleotides 2-7) and exploits its functions (90). Of note, evidence that the 3′ UTR is the sole target of miRNA interactions is also changing, as some recent studies have demonstrated that miRNA binding sites can occur both in 5′ UTR and within the coding sequences (91).
The canonical role of miRNAs is targeting mRNA repression, that can be the result of either reduced translational efficiency or an actual decrease in the mRNA levels. Degree of complementarity between the miRNA and the target regions in the mRNA is thought to have a role where sufficient complementarity is believed to result in mRNA degradation while less complementarity leads to translational inhibition (86).
Eventually, miRNAs form complex networks of interactions, as one miRNA can regulate > 100 mRNAs, and one mRNA can be targeted by many different miRNAs (92).
Most studies report miRNA role in gene inhibition, but recent research has demonstrated that miRNAs are capable to perform dual roles, since they can also trigger RNA activation (RNAa), interacting with the promoter sequence of a target gene at nuclear level, improving occupancy of transcription factors and RNA Pol II, eventually activating target transcription (91). Interestingly, Turner and colleagues also demonstrated that in some cases miRNAs can also trigger autoactivation (93). In this context the re-import of miRNAs into the nucleus is especially relevant, and AGO2, importin8 (IPO8), Exportin-1 (XPO1) and the P-body-associated RNAi factor, TNRC6A, have been identified as the main actors for miRNA shuttling between cytoplasm and nucleus (88, 90, 91, 94).

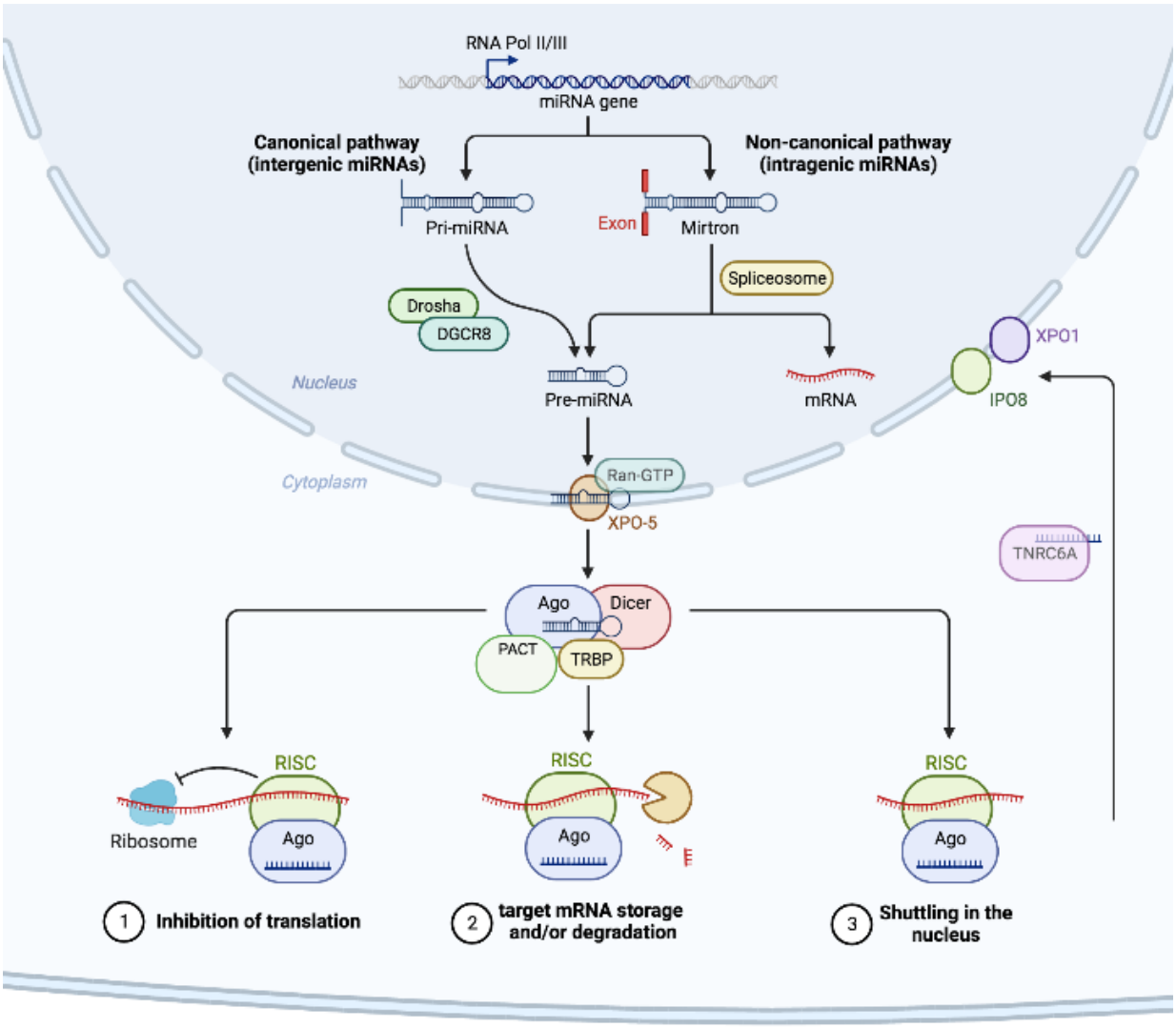


Figure 6. MiRNA biogenesis. Adapted from "miRNA Processing Mechanisms in the Brain", by BioRender.com. Retrieved from https://app.biorender.com/biorender-templates

1.2.2 Muscle-specific miRNAs (MyomiRs)

MiRNA expression profile varies across tissues, since it is driven and modulated by several tissue-specific epigenetic mechanisms. Based on this, conventional criteria have been proposed to classify miRNAs, depending on tissue abundance differences. Therefore, miRNAs have been divided into "tissue-enriched" and "tissue-specific", depending on whether they were detected at <20-fold or ≥20-fold levels in the tissue evaluated, compared to the mean values for other tissues, respectively (95). Recently, the application of a Tissue Specificity Index (TSI) on wide-transcriptome data has allowed the classification of miRNA genes into housekeepers, "intermediate" and tissue-specific. According to these notions, muscle-specific miRNAs, conventionally called myomiRs, include miR-206, -133a, -133b, - 1, -208a, -208b, and -499a, with the exceptions of miR-206, being skeletal muscle-specific, and miR-208a, having a cardiac muscle-restricted expression (96).
It should be kept in mind that other non-muscle specific miRNAs might either influence the myogenic processes or be involved in other phenotypic changes associated with skeletal muscle environment, such as mitochondrial function, metabolism, or hypertrophy (96). Indeed, 44 miRNAs, including myomiRs, appeared to be altered during differentiation of primary human skeletal muscle (97), whereas, in another study, up to 60 miRNAs resulted differentially expressed (98). Some myomiRs are monocistronic and transcribed independently, on the other hand others are organized as clusters

with genomic colocalization and transcribed as bicistronic or polycistronic transcripts, such as miR-1-1/miR-133a-2, miR-1-2/miR-133a-1 and miR-133b/206. Interestingly, they also share similar sequences and may modulate a set of similar, but not identical, target genes (96).

MyomiR expression follows a distinct spatio-temporal pattern, aiming to properly regulate myogenesis. Skeletal myogenesis refers to the process of muscle development, whereby myogenic precursor cells can differentiate and fuse to form myofibers. Briefly, precursor cells undergo proliferation and differentiation, while a subset remains capable of self-regeneration, repairing tissue injuries in adulthood. Specifically, the crucial steps of skeletal muscle development involve: activation and proliferation of stem cells leaving the cell cycle and differentiating into myoblasts; myoblast alignment and fusion to form multinucleated myocytes; terminal differentiation of myocytes into myofibers. Satellite cells, that are muscle stem cells in adults, are associated with healing and regeneration of muscle. All aspects of muscle development are regulated by transcription factors known as myogenic regulation factors (MRFs). Several MRFs are characterized by the basic helix-loop-helix (bHLH) motif and include myoblast determination protein (Myod), myogenic factor 5 (Myf5), and 6 (Myf6, also called MRF4), Myog and the serum response factor (SRF). Others contain the MADS-box motif and include the myocyte enhancer factor-2 (Mef2) family of transcription factors. In addition, in a quiescent status, satellite cells express paired box transcription factor 7 (Pax7) and, to a smaller extent, 3 (Pax3). Under physiological conditions, satellite cell activation is determined by co-expression of Pax7, Myod, and Myf5. Myogenic differentiation is then triggered by the suppression of Pax expression, allowing for exclusive expression of MRFs, specifically Myod that is required for myogenic identity establishment in the early stages, and Myog during terminal differentiation events (*Figure 7*). Interestingly, myomiR expression controls and, in turn, can be regulated by MRFs (95).

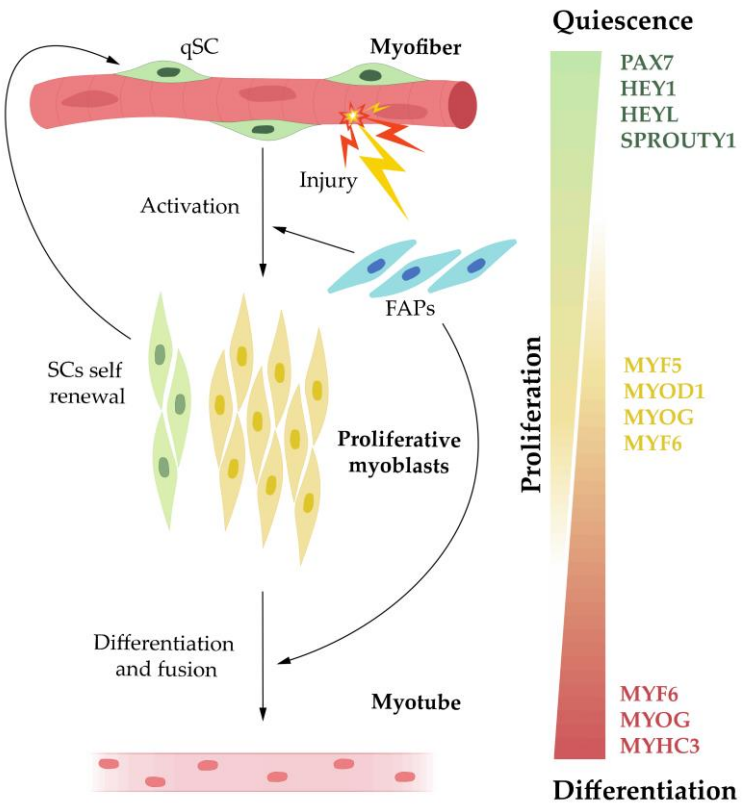


Figure 7. Main phases of myogenesis in skeletal muscle homeostasis, including the mainly myogenic regulatory factors (MRFs) involved. SCs: satellite cells; qSC: quiescent satellite cell; FAPs: fibro-adipogenic progenitors. *Adapted from Aránega AR et al., 2021.*

1.2.2.1 MiR-206

It is generally accepted that miR-206 and miR-1 play a crucial role in muscle differentiation. Indeed, miR-206 is considered to be a member of the miR-1 family but differs from miR-1 by four nucleotides outside of the seed region (99). Not surprisingly, there is some redundancy in miR-1 and miR-206 functions (100). During C2C12 myoblast differentiation into myotubes, miR-206 expression is significantly upregulated. In particular, miR-206 overexpression blocks cell cycle progression and induces myotube formation, whereas its inhibition produces opposite results (101). Interestingly, it has been reported that miR-206 expression is higher in muscle composed primarily of slow-twitch/oxidative fibers compared to fast-twitch/glycolytic fibers suggesting that miR-206 may be fiber type I specific (102).
Pax7 and Pax3 are direct targets of miR-206, which inhibits their expression to promote myoblasts transition from proliferation to differentiation (103). Also, DNA polymerase alpha 1 (Pola1) mRNA degradation is orchestrated by miR-206, in order to withdraw the quiescent stage of cells and mediate muscle differentiation (101). Myod, Myog, and Mef2 bind to the upstream regions of the miR-206 locus, ensuring its skeletal muscle tissue-specific expression. For example, it has been shown that Myod induces miR-206 to inhibit utrophin (Utrn) and follistatin-like 1 (Fstl1) post-transcriptionally, that are molecules able to maintain the proliferating status of myoblasts (104). Moreover, miR-206 promotes the function of Myod, thus establishing a positive regulatory feedback loop. Another way for miR-206 to promote differentiation is through inhibition of Histone deacetylase 4 (HDAC4), responsible for chromatin remodeling and repressor of myoblast differentiation via Mef2a inhibition (99). Furthermore, since the Mef2a transcription factor has been reported to promote the transformation of type II fast glycolytic fibers into type I slow oxidative fibers, miR-206 might have a role in determining the fiber type switch (105). On the contrary it has been described that Mef2c stimulates the expression of myomiRs, specifically miR-206 and miR-1, resulting in myoblast differentiation (106). Studies have also shown that TGF-β regulates muscle differentiation through inhibition of miR-206 expression, but miR-206 is capable of counteracting this inhibition by generating a regulatory loop that suppresses TGF-β induction (106, 107). Finally, in myoblasts, TARDBP physically associates with miR-1 and miR-206, but not with the miR-133 family, thus disrupting their availability for incorporation into RISC (106).

1.2.2.2 MiR-133a/b

The effects of miR-133a/b on myogenesis are still debated, but they surely play an important role in this process, since it was demonstrated that their expression is massively induced at the beginning of myogenesis in human myoblasts (108). Previous observations have indicated that miR-133 primarily promotes the proliferative state of myoblasts by repressing SRF in C2C12 cells (109). More recently, evidence suggests that miR-133 instead could also act by inhibiting myoblast proliferation to promote muscle cell differentiation targeting of pro-proliferative target genes (110). Moreover, via Myog induction, IGF1 stimulates miR-133, which represses IGF-1R by negative feedback, thus accelerating muscle differentiation (111). Differences in those studies highlight the complexity of miRNA targeting events and could suggest that miR-133 is able to influence either proliferation or differentiation in a context-dependent manner. Interestingly, it has been proposed that miR-133a mediates mesenchymal lineage commitment by inhibiting differentiation into osteoblasts and chondrocytes in order to control skeletal muscle development. In addition, it was established that miR-133a/b mediate the ability of muscle cells to enter the adipocyte cell line during muscle regeneration (106). Of note, a novel regulatory circuitry has been recently identified, in which coding and non-coding RNAs can regulate each other's expression, hence the name competing endogenous RNA (ceRNA) (112). According to this idea, a long noncoding RNA (lncRNA) linc-MD1 acts as a binding "sponge" for miR-133 downregulating its abundance, and thereby modulating myomiR

mRNA targets as well, including Mef2c (112). Lastly, a Myod-miR-133a-UCP2 axis has been involved in muscle differentiation resulting in a UCP2 downregulation that alleviates the developmental repression (113).

1.2.2.3 MiR-1

Paradoxically, although originating from the same miRNA polycistronic transcript, miR-1 and miR-133 become two independent, mature miRNAs, inhibiting different target genes and exerting opposing effects to skeletal muscle development (114). Indeed miR-1, activated by MYOD, promotes muscle differentiation by inhibiting HDAC4 (109). The same biological results are obtained by the positive feedback loop where both miR-1 and miR-206 inhibit PAX7 which in turn, by inhibiting DNA binding 2 (ID2), is no longer able to downregulate MYOD, which keeps promoting miR-1 and miR-206 expression (106). Similarly to what observed with miR-133, a crosstalk between IGF1 and miR-1 has been identified. In particular, they reciprocally regulate one another via the IGF1–AKT–FOXO3–miR-1 axis during C2C12 differentiation (115). Interestingly, it has been reported that miR-1 efficiently enters skeletal muscle mitochondria during muscle development whereby it stimulates the translation of specific mitochondrial genome-encoded transcripts, promoting myogenesis in mice (116, 117).

1.2.3 MyomiR in ALS, SMA, and SBMA

In skeletal muscle tissue, myomiR regulatory loops are targeted in diseases where skeletal muscle undergoes atrophic events, and myomiRs both regulate and are regulated by these processes (118). Several myomiR-related pathophysiological mechanisms during atrophy and reinnervation attempts, which are potential targets for further therapeutic interventions, have been identified by using denervation models that finely recapitulate some atrophic features of MNDs. For example, a study performed in a denervated rat model allowed to decipher the time course of myomiR expression in muscle after denervation, confirming that miR-206, miR-133a, and miR-1 levels were time dependent: indeed, following denervation, miR-133a and miR-1 were downregulated but both myomiR expressions increased after 4 months during reinnervation. On the other hand, miR-206 levels were persistently increased 1–4 months after reinnervation, but not right after denervation. Four months after nerve scission, the re-innervated muscle was predominantly type II glycolytic fibers, suggesting that miR-206 may aid the determination of fiber type via down-regulation of MEF2 transcription factor activity (105). In addition to this evidence, it was demonstrated that in miR-206 null mice, but not in miR-133b null mice, re-innervation was impaired following nerve injury (119). Similar evidence was obtained by the work of Williams and colleagues, where miR-206 has been found to promote formation of new NMJs following peripheral nerve denervation (120). Overall, this data strongly suggests that miR-206 has a crucial role in regulating NMJ repair following nerve injury, being a key molecule of bidirectional signaling between skeletal muscle fibers and nerves. Indeed, several evidence demonstrated that miR-206 is involved in modulating skeletal muscle mass as well. For example, miR-206 transcript is significantly increased during skeletal muscle hypertrophy (102). In addition, miR-206 is highly expressed in newly formed muscle fibers, indicating that miR-206 may be involved in muscle regeneration and maturation in muscle atrophy (121). However, it must be noticed that discrepancies exist regarding the unique role of miR-206 in skeletal muscle. Indeed, genetic deletion of miR-206 negatively affected muscle post-embryonic regeneration following denervation or cardiotoxin injury (120, 122). On the contrary, it was also demonstrated that deletion of the miR-206/miR-133b cluster was dispensable for proper muscle regeneration (123). The authors suggest that these differences and the absence of a robust phenotype could be explained by subtle differences in the genetic background and/or mouse strain but may also

reflect a function redundancy within the myomiR family, in particular due to overlapping functions of the related miR-1/133a clusters. Thus, miR-1 and miR-133a could compensate for the loss of miR-206/133b expression loss. In support of this idea, the absence of miR-206 in D. melanogaster, which has only one genomic copy of miR-1, is coincident with severely deformed musculature in miR-1 mutant larvae (123). Of importance, local injection of a cocktail of double-stranded miRNAs containing miR-206, miR-1, and miR-133 has been shown to up-regulate Pax7, Myod, Myog, enhancing muscle regeneration and effective fibrosis prevention in a rat skeletal muscle injury model (124).

In the ALS context, miR-206 knock out (KO) SOD1 mice showed a lower survival rate and faster disease progression compared to SOD1 littermates, despite similar disease onset (119, 120). Moreover miR-206 had been shown to be increased in skeletal muscle tissue of ALS patients with a genetic background (125, 126) and, interestingly, patients with SOD1-mutations presented a stronger miR-206 upregulation compared to C9orf72-, sALS patients and controls (126), revealing that subtle differences in disease etiopathogenesis may affect miRNA expression among patient subgroups. Regarding miR-1 and miR-133, they resulted downregulated both in SOD1 mice and ALS patient biopsies, indicating that both muscle proliferation and differentiation are compromised (127, 128).

Several studies showed a close relationship between miRNA alteration and SMA. It was demonstrated that, the multiprotein SMN complex assembles miRNA-ribonucleoprotein complexes (miRNPs) and transcriptosomes. Mice specifically lacking the miRNA-processing enzyme Dicer in MNs, have been reported to display hallmarks of SMA (129). Moreover, miR-206 is upregulated in Δ7SMA mouse quadriceps, along with a decrease in the percentage of type II fibers and an increase in atrophic muscle fibers. Furthermore, a strong upregulation of the miR-206-HDAC4-FGFBP1 pathway has been observed in the late phase of the disease, suggesting a critical role of the miRNA in myogenic processes in SMA (130).

Although abnormal miRNA expression patterns have been proposed to be related to the etiology and progression of poly Q diseases, there is an evident paucity of information regarding miRNA role in SBMA and no investigation at all on myomiR involvement in the disease.

1.3 MiRNA-based therapeutic options

The lack of effective therapies for MNDs emphasizes the need for alternative treatment approaches. Targeting skeletal muscle for therapeutic intervention has attractive advantages over targeting the CNS. Drug delivery to muscle is much easier than to the spinal cord. Moreover, tissue sampling of skeletal muscle is readily available, allowing for direct analysis. Finally, secondary side effects associated with CNS could be bypassed (76). For example, AR suppression via ASO delivery by intramuscular administration in SBMA mice did not reduce circulating testosterone levels (77). Drugs targeting the skeletal muscle of patients with LMN impairment could prevent NMJ dismantling and allow sprouting and formation of new synapses, ultimately improving the patient's quality of life (131).

However, the previously described heterogeneity given by non-cell autonomous pathogenic mechanisms implicated in MNDs, therefore the involvement of different tissues, might require pharmacological multi-target strategies. The branch of miRNA-based therapeutics is a developing field in comparison to other oligonucleotide-based therapeutics such as siRNA or ASO (132-134), some of whom are already FDA-approved for MND treatment (i.e. nusinersen) (135). Several preclinical and clinical results have been obtained in MNDs however, they are mainly focused on targeting the CNS (136, 137). There are two main attractive advantages of miRNA-based therapeutics. First, mature miRNAs are often nicely conserved across multiple vertebrate species. Therefore, one miRNA can turn out to be potentially targeted in both preclinically and clinical trials (138). In addition, one miRNA can target multiple mRNAs, resulting in a variety of signaling pathway regulation, which differs from traditional drug mechanisms of action where one drug usually interacts

with one molecular target. On the other side, for the same reason, the on- and off-target toxic effects must be systematically mapped to ensure the specificity and the tolerability of miRNA restoration strategies to circumvent any undesirable results, such as innate immune responses, which cause significant challenges in clinical applications. However, being smaller in size than ASOs, miRNAs should be safe and well tolerated in human patients and should be less likely to elicit an immunogenic response (76).

To date, two miRNA-based strategies have been formulated. The first one is the restoration of suppressed miRNA levels by double strand miRNA mimic administration (agonist) which is processed at the nuclear level as endogenous miRNA, before exploiting its functions. The other is inhibiting miRNA function by using single strand anti-miR (antagonist) to repress overactive miRNA function (139). There are good prospects and challenges (i.e avoiding nuclease degradation) when it comes to miRNA delivery route.

The main two are viral-based and non-viral-based systems. The advantage of the viral-based systems (retrovirus, lentivirus, adenovirus and AAV) are the high transfection efficiency and continuous expression of the agonist or antagonist miRNAs. Whereas the non-viral systems have lower immunogenicity and toxicity but lower transfection efficiency (140). Novel non-viral systems include attractive options like exosomes, PEGylated liposomes, lipidoids, and biodegradable polymers (141). The incorporation of miRNAs into these systems can increase the quantity and quality of miRNA packed and delivered to the desired sites of action with a better bioavailability, compared to "naked-miRNA" delivery, hence reducing doses; plus, engineered nanoparticles can selectively deliver to specific cell population, which will finely help to achieve the therapeutic goal (142).

The majority of studies implying ALS miRNA-based treatment has been performed targeting the CNS. First promising results have been provided by an anti-miR-155 treatment of G93A-SOD1 mice that displayed a 10-day prolonged survival after therapy (143). Increased lifespan in ALS mice was also achieved after miR-17~92 delivery, together with motor deficit improvements (144), and by a single intracerebroventricular antagomiR-29a injection (145).

In the SMA landscape, similar treatments were conducted on SMA mice. For example, anti-miR-181a-5p injections into the cerebral lateral ventricle significantly improved mouse survival (146).

So far, only two studies have been performed with the objective of testing miRNA AAV vector–mediated delivering for therapeutic purposes. Those non-muscle specific miRNAs are miR-298 and miR-196a. In particular, miR-298 downregulated AR mRNA and protein levels, hence decreasing its toxicity both in cells overexpressing wild type and mutant AR, or fibroblasts derived from SBMA patients. Moreover, miR-298 intravenous administration in SBMA mice resulted in the amelioration of muscle atrophy (147). On the other hand, miR-196a -found to be overexpressed in the symptomatic stage in spinal cords of SBMA mice, probably as a protective mechanism- binds to CELF2 and represses it, resulting in a lower amount of mutated androgen receptor with SBMA motor improvement (148). Therefore, it was suggested that that early administration of miR-196a can represent a new therapeutic approach.

1.4 MiRNA as disease biomarkers

Biomarkers are genetic, molecular, or biochemical components that can be quantifiable, reproducibly measurable with low variation coefficients, to facilitate the identification and analysis of pathological processes, when expressed in the human body (149). Depending on the purpose, biomarkers can broadly fall under five categories: diagnostic -when able to discriminate disease phenotype prior to diagnosis- or prognostic -prior to treatment initiation-; predictive -when they are designed to predict treatment response prior to treatment initiation- or disease progression -if they are able to measure treatment response-; pharmacodynamic -if they are markers after treatment and capable, for example, to show whether the therapeutic target is restored after therapy- (150).

The monogenic nature of SMA or SBMA and genetic tests, currently make novel diagnostic markers not a priority. However, as aforementioned, disease progression is variable, and tools to provide early and differential diagnosis or predict disease course would be beneficial (72).

A single biomarker may be useful but not fulfill insights into all areas, such as rating disease severity and progression, improving phenotype classification and patient stratification and eligibility for clinical trials, helping in the development of therapeutic strategies or examining the variability in drug responsiveness. For example, SMN mRNA and protein levels can provide insight into current disease state but it has been demonstrated that they are not always effective for monitoring disease progression and response to therapy since they neither change over time, nor correlate with disease severity and are inconsistent between the periphery and the central nervous system (150). Thus, a combination of robust biomarkers may provide greater insight and more accurate clinical assessment to avoid, among others, side effects such as long-term exposure to expensive drugs with still unknown long-term drug-related adverse events (150).

To date, the neurofilament, which increases upon axonal degeneration, is one of the most promising biomarkers in ALS. The levels of phosphorylated heavy and light neurofilaments are detected in the CSF and serum respectively, and the neurofilament light chain level is also considered a good prognostic marker for ALS, with high levels in the CSF corresponding with short survival. Interestingly, neurofilaments and CSF proteomic profile, although not yet validated, have recently drawn attention as promising biomarkers in SMA (151, 152). Although highly reliable because it is protected by the blood-brain barrier, it must be noted that CSF extraction is not easily suitable for primary screening and monitoring purposes, owing to its invasiveness (lumbar puncture is required). Another prognostic biomarker for ALS survival is the serum creatine kinase, and it was also studied as predictive and pharmacodynamic biomarker for SMA (151), as well as SBMA as previously mentioned (79). Finally, since abnormal aggregation of TDP-43 is a pathological hallmark of the disease, determining specific TDP-43 variants to provide additional ALS biomarkers has recently been proposed.

Despite numerous efforts, the number of reliable biomarkers for MNDs is still limited and, specifically, the identified molecules are not able to stratify patients' phenotypes.

MiRNAs possess several characteristics that make them suitable biomarkers for MNDs. Because of their resistance to degradation by RNases, they are stable in body fluids such as serum, plasma, CSF, saliva and urine. They are able to traverse the blood–nerve barrier and be actively secreted and circulated in the bloodstream during tissue structural rearrangements, reflecting specific biological states of a tissue or organ, hence they are associated with distinct pathophysiological conditions. Moreover, miRNA analysis is minimally invasive and cost-effective (151).

MyomiRs have been extensively investigated as ALS and SMA biomarkers, because their expression may reflect the state of the muscle tissue (153, 154). For instance, miR-206 is found to be upregulated in ALS sera compared to healthy controls (153). A recent study has shown a correlation between ALS disease stages and serum miR-206 expression, revealing miR-206 upregulation during the initial ALS stages and a downregulation in the late phase (155). MiR-206 levels are also higher in the sera of spinal-onset ALS compared to bulbar patients, because of the miRNA downregulation associated with very rapid atrophic process in bulbar patients (156). MiR-133a is another potential biomarker, because its downregulation during ALS progression has been reported (155), whereas in the SMA context miR-133a was suggested to predict patients' motor function response to nusinersen therapy (154). In addition, miR-1 and miR-133a/b are upregulated in sera of spinal-onset patients compared to levels in bulbar-onset ALS patients and controls (156, 157). Finally, miR-1 has been found to be upregulated in sera from both sALS and fALS patients compared to that in controls (157).

All these studies highlight the potential of circulating miRNAs, along with other molecules (e.g. peripherins or neurofilament light chain), as easily accessible biomarkers especially for MND diagnosis and prognosis, improving patient stratification and therapeutic follow-up.

However, one must consider the external and internal factors that may influence miRNA levels, ranging from genetic variation, race, gender, inflammatory status, and lifestyle factors to differences

in methodology, such as the techniques used to process samples and measure the miRNA levels. Also, blood samples are indeed easily obtained at low-cost/low-risk and less operator-dependent. However, there is a chance of additional factors that contribute to what is found in the blood other than from the brain, due to blood circulation.

For instance, it has been demonstrated that the concentrations of miRNA levels may vary in serum and plasma samples within the same individual, and a research study showed that different miRNAs with variable concentrations were found across blood, plasma, serum, or exosome samples. In addition, data analysis could also affect the statistical significance of miRNA levels in biomarker studies (158).

Despite the outlined limitations, miRNA potential as biomarkers is a continuously evolving research field. MiRNAs could become a routine approach in the development of timely and personalized patient profiles, with all the advantages already depicted.

2. AIM

MNDs comprise a spectrum of neurodegenerative disease affecting upper and lower MN, resulting in muscle weakness. Currently, there are no effective treatments for MND, highlighting the need to obtain a deeper understanding of the molecular events underlying degeneration of both MNs and muscle tissue, with the aim of developing successful therapies. Muscle tissue is enriched in a group of microRNAs called myomiRs, which are effective regulators of muscle homeostasis, plasticity and myogenesis in both physiological and pathological conditions.

To this purpose, the aims of the PhD project are:

- To identify muscle-specific microRNA (myomiR)/mRNA target pairs differentially expressed in skeletal muscle of ALS, SMA and SBMA animal models during disease progression.

- To demonstrate myomiR role as noninvasive biomarkers for ALS, SMA and SBMA.

3. MATERIALS AND METHODS

3.1 Animal Models

All animal experiments were carried out in accordance with the EU Directive 2010/63 and with the Italian law (D.L. 26/2014) on the protection of animals used for scientific purposes. Transgenic G93A-SOD1 (B6SJL-Tg (SOD1*G93A)1Gur/J) [MGI: 2183719] and control B6.SJL mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA, USA), maintained and bred at the animal house of the Fondazione IRCCS Istituto Neurologico Carlo Besta in compliance with institutional guidelines. The project was approved by the Ethics Committee of the Institute and the Italian Ministry of Health (ref. IMP-01-12; 183/2018-PR: date 03/2018-10/2021). Transgenic G93A-SOD1 progenies were identified by quantitative real-time PCR (qPCR) amplification of the mutant human SOD1 gene as previously described (34). Only G93A-SOD1 male animals carrying more than 27 mutant SOD1 copies were included in the study and sacrificed for tissue collection at week 7–8–10 (presymptomatic stages), week 12 (onset), week 15 (symptomatic stages), and week 18 (late stage) (159) by exposure to CO_2. Age- and sex-matched healthy littermates were used as controls.
The Δ7SMA (stock no. 005025; Jackson Laboratory, Bar Harbor, Maine, USA) [MGI: 109257] were transgenic heterozygous mice (Smn+/−, hSMN2+/+, SMNΔ7+/+). All animal experiments received approval by the Italian Ministry of Health review board (1007/2016-PR and 96/2016-PR: date 2016-2019). Pups were identified by genotyping, as previously reported (160). SMNΔ7+/+ male mice were euthanized for tissue collection at day 2 (early disease stage) and day 10 (late disease stage). Because Δ7SMA mouse lifespan is very short (13.3–15 days) and the onset of the disease is very difficult to identify (161), two time points were selected. Age-matched healthy littermates were used as controls.
KI-SBMA model [MGI: 88064] was developed by Lieberman laboratory (162). Animal care and experimental procedures were conducted in accordance with the institutional guidelines (Università degli Studi di Milano), and the project was approved by the Italian Ministry of Health (423/2015-PR: date 2015-2018). In these mice, a portion of the coding region of mouse Ar exon 1 (Pro37 to Gly423) was exchanged for the same region in human AR exon 1, introducing 113 CAG repeats. Hemizygous female mice carrying AR113Q in the X chromosome were crossbred with C57Bl/6J male mice to maintain the colony. Generation and genotyping of AR113Q knock-in mice was conducted as previously described (162, 163). Male animals were sacrificed for tissue collection at weeks 8 (presymptomatic stage), 14 (onset of disease), and 40 (symptomatic phase of disease) (162) by exposure to CO_2. Disease stage was confirmed by monitoring body weight, rotarod performance test, and the grip strength of the animals.
Denervation animal models were three-month-old male mice (C57BL/6 strain), anaesthetized with ketamine and xylazine (respectively 90 mg/kg IP and 12.5 mg/kg IP). An incision was made through the skin and the upper region of the left gluteal muscle to expose the sciatic nerve, which was then cut 1–2 mm distal to the sciatic notch. The proximal portion of the nerve was sutured to prevent errant reinnervation of the gastrocnemius muscle. Right sciatic nerve was only exposed and utilized as sham internal control in each animal. C57BL/6 strain not subjected to denervation was included as control. Mice were sacrificed 7 and 21 days later.

3.2 Quantitative Real-Time PCR to Assess MyomiR Expression in Mouse Muscle Tissues

At sacrifice, gastrocnemius muscles were collected, snap frozen, and maintained at –80°C until use. Total RNA was extracted with Trizol reagent from 100–200 mg of muscle tissues and reverse-transcribed to cDNA using TaqMan microRNA Reverse Transcription Kit (Thermo Fisher Scientific Inc., Foster City, MA, USA) with specific primers for miR-206, miR-133a, miR-133b, and miR-1. cDNA aliquots corresponding to 15 ng total RNA were amplified by quantitative real-time PCR in duplicate, with Universal PCR Master Mix and specific predesigned TaqMan MicroRNA assays (Thermo Fisher Scientific Inc.). U6, which was stably expressed across the G93A-SOD1, Δ7SMA, AR113Q, and control muscle tissues (as shown by standard deviation of Ct values < 0.5; Ct range: from 22.91 to 23.07), was used as endogenous control for data normalization. The range of Ct values for the miRNAs across the MND mouse muscle samples were the following: (i) miR-206, from 18.32 to 24.43; (ii) miR-133a, from 16.65 to 21.34; (iii) miR-133b, from 16.56 to 28.77; (iv) miR-1, from 17.14 to 23.56. The range of Ct values for the miRNAs across the control samples were the following: (i) miR-206, from 20.67 to 26.82; (ii) miR-133a, from 16.58 to 23.60; (iii) miR-133b, from 17.04 to 28.35; (iv) miR-1, from 17.50 to 32.20. MiRNA levels normalized to U6 were expressed using the formula $2^{-\Delta Ct}$. To represent the expression of myomiRs and their predicted targets, we first calculated the $2^{-\Delta Ct}$ by normalizing myomiR and target expression towards U6 and 18S respectively; fold change was estimated by dividing the $2^{-\Delta Ct}$ of pathological versus $2^{-\Delta Ct}$ control values of mice. Then, the $\log_2$ of fold change was shown in Figure 4.

3.3 MyomiR Target Gene Prediction and Analysis in Mouse Muscle Tissues

MyomiR targets were predicted *in silico* by the miRWalk and miRBase database using the default score parameters (164), and from the literature, selecting genes known to be specifically expressed in skeletal muscle and to regulate the fundamental processes of myogenesis and regeneration (99, 106, 109, 165-169). For gene expression analysis, 0.5ng of total RNA, previously examined for myomiR expression, was treated for 15 min at room temperature with 1U of DNaseI (Merck, Darmstadt, Germany), and reverse-transcribed to cDNA using the HighCapacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc.), according to the manufacturer's instructions. qPCR was performed using the CFX 96 Real-Time System (Bio-Rad Laboratories, Segrate, Italy) in a 10 µl total volume, using the iTaq SYBR Green Supermix (Bio-Rad Laboratories), with a final concentration of primers of 500 nm, and with cDNA aliquots corresponding to 10 ng of total RNA. Primers for the selected genes, reported in Table 1, were designed using the program Primer 3 Plus and purchased from Eurofins Genomics (Ebersberg, Germany). Data were normalized to the housekeeping gene "ribosomal protein lateral stalk subunit P0" (Rplp0) and expressed as fold changes using the formula $2^{-\Delta Ct}$.

Rplp0	5′-GGT GCC ACA CTC CAT CAT CA-3′ (forward)
	5′-AGG CCT TGA CCT TTT CAG TAA GT-3′ (reverse)
Pax7	5′-GTA TGG CCA AAC TGC TGT TGA T-3′ (forward)
	5′-GGA GTG TTC CCC AAG CTT CA-3′ (reverse)
Myod1	5′- GGC TAC GAC ACC GCC TAC TA-3′ (forward)
	5′-GTG GAG ATG CGC TCC ACT AT-3′ (reverse)
Myog	5′-GGG CAA TGC ACT GGA GTT-3′ (forward)
	5′-CAC GAT GGA CGT AAG GGA GT-3′ (reverse)
Mef2a	5′-CAG CCA GCT CAA CAT TAG CA-3′ (forward)
	3′-GGC ACC GTG AGG TCT GTA GT-3′ (reverse)

Table 1. List of primer sequences of the selected myomiR target genes. Primers were used in qPCR reactions to assess the expression of the putative target genes of myomiRs, *Pax7*, *Myod1*, *Myog* and *Mef2a*, and the housekeeping *Rplp0*.

3.4 Western Blotting Assay

For total protein extraction, 50 mg of muscle tissue were homogenized in lysis buffer, phosphate-buffered saline (PBS) pH 7.4, supplemented with 1% Nonidet P-40 protease inhibitor cocktail (Merck), phosphatase inhibitors (sodium vanadate 100 mM and sodium fluoride 100 mM), and EDTA (1 µM), with Tissue Lyser II and stainless steel glass beads (Qiagen, Venlo, Netherlands). Crude extracts were centrifuged for 10 min at 5000 rpm at 4°C to remove particulate matter. Supernatant protein concentration was determined by the bicinchoninic acid method (BCA assay, EuroClone, Pero, Italy). Western immunoblot analysis was performed on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis loading 15 µg of total proteins. Samples were then electrotransferred to nitrocellulose membranes 0.45 m (Bio-Rad Laboratories) using a semidry transfer apparatus (Trans-Blot® Turbo™ Transfer System, Bio-Rad Laboratories). Nitrocellulose membranes were treated overnight with a blocking solution containing 5% nonfat dry milk in TBS-T (Tris-buffered saline with 0.1% Tween 20) and then incubated with the right primary antibody overnight at 4°C (anti-GAPDH dilution 1:10000, Immunological Science, Rome, Italy, MAB-10578; anti-PAX-7 dilution 1:1000, Thermo Fisher Scientific Inc., PA-1-117; anti-MYOG dilution 1:500, Thermo Fisher Scientific Inc., MA5-11486; anti-MEF2A dilution 1:1000, Thermo Fisher Scientific Inc., PA5-27344). Immunoreactivity was detected using secondary peroxidase-conjugated antibodies: goat anti-rabbit (Jackson ImmunoResearch, Cambridgeshire, UK, dilution 1:5000) was used to identify anti-MEF2A; goat anti-mouse (Jackson ImmunoResearch, dilution 1:5000) was used to identify all the other primary antibodies. Original representative images of western blots were reported in Figure S5. The immunoreactive regions were then visualized using the enhanced chemiluminescence detection kit reagents (Westar Antares, Cyanagen, Bologna, Italy). A ChemiDoc XRS System (Bio-Rad Laboratories) was used for the image acquisition. Optical intensity of samples assayed was detected and analyzed using the Image Lab software (Bio-Rad Laboratories).

3.5 Histological Analysis

Gastrocnemius muscles were obtained from three G93A-SOD1 mice at week 18, three Δ7SMA mice at day 10, three AR113Q mice at week 40, and respective age-matched controls. Samples were immediately frozen in isopentane precooled in liquid nitrogen for histological analyses and stored at −80°C until use. For each animal, three 10 µm thick frozen tissue sections were stained with hematoxylin-eosin (Bio Optica, Milan, Italy) and examined by optical microscopy (Nikon GMBH, Germany) at 40× magnification. Muscle fiber diameters were measured using Image Pro-Plus (Media Cybernetics, Silver Spring, MD, USA). Fiber diameter was defined as the widest transversal distance. For each hind limb muscle section examined, fiber diameters were measured in three randomly selected microscope fields.

3.6 Patients and Biological Samples

A cohort of 47 clinically defined MND patients was enrolled for the study, including: 14 ALS SOD1 mutant-patients (8 sALS and 6 fALS) [OMIM: #105400 Amyotrophic Lateral Sclerosis, ALS1]; 23 pediatric SMA patients [17 SMA type II [OMIM: #253550 Spinal Muscular Atrophy, type II, SMA2) and 6 SMA type III (OMIM: #253400 Spinal Muscular Atrophy type III, SMA 3)], 10 SBMA patients [OMIM: #313200 Spinal and Bulbar Muscular Atrophy, X-linked 1, SMAX1] followed-up at Neurology III Unit, Neurology IV Unit, Developmental Neurology Unit, Neurology X Unit respectively, and genetically assessed at Unit of Medical Genetics and Neurogenetics at Fondazione IRCCS Istituto Neurologico Carlo Besta (Milan, Italy). Seventeen sex and age-matched healthy adults were included as controls for the ALS and SBMA patients, whereas nineteen pediatric patients affected by encephalitis associated to antibodies anti-NMDA receptor, whose pathogenesis has no overlap with MNDs, were considered as disease controls for children with SMA type II and III due to difficulty in collecting biological samples from healthy children. Patients' clinical features are reported in Table 3. The study was performed in accordance with the ethical standards of The Code of Ethics of the World Medical Association (Declaration of Helsinki). The investigation and use of patients' data for research purposes were approved by the Ethics Committee of Fondazione IRCCS Istituto Neurologico Carlo Besta (project identification code 92/2019: date January 2019–January 2022), in accordance with the Declaration of the World Medical Association. Written informed consent was obtained from each subject or legal representative in case of pediatric subjects. Biological samples were stored at −80°C in the Biobanks of Fondazione IRCCS Istituto Neurologico Carlo Besta and IRCCS Istituto di Ricerche Farmacologiche Mario Negri (Milan, Italy) until use.

3.7 Quantitative Real-Time PCR to Assess MyomiRs in Patient Serum Samples

From 250 µL of serum, total RNA was extracted by using the miRNeasy serum/plasma kit (Qiagen), and reverse-transcribed to cDNA using TaqMan MicroRNA Reverse Transcription Kit with specific primers for miR-206, miR-133a, miR-133b, miR-1. MiR-16 was used as endogenous control (170) since it was stably expressed in serum from patients (as shown by standard deviation of Ct values < 0.5 and Ct range across samples between 28.90 to 29.20). cDNA aliquots corresponding to 15 ng total RNA were amplified by qPCR in duplicate using Universal PCR master mix and specific predesigned TaqMan MicroRNA assays (Thermo Fisher Scientific Inc.). MiRNA levels were normalized to miR-16 and expressed using the formula $2^{-\Delta Ct}$.

3.8 Statistical Analysis

By Shapiro–Wilk test, data resulted nonparametrically distributed and were then analyzed by Mann–Whitney test for comparison of two independent groups. p-values < 0.05 were considered as statistically significant. The miRWalk 2.0 and the miRBase databases were employed to predict myomiR putative target genes. Nonparametric Spearman correlation test was applied to evaluate the correlation between the expression levels of each miRNA and its target genes in muscle tissues of MND animal models during disease progression. Receiver operating characteristic (ROC) curves were used to assess sensitivity and specificity of miR-206 in serum samples as biomarker able to discriminate between MND patients and controls. GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA) was used for data elaboration and statistical analysis.

4. RESULTS

4.1 Dysregulated myomiR expression in G93A-SOD1, Δ7SMA, and AR113Q, mouse muscle tissue during disease progression

To verify whether myomiR dysregulation in skeletal muscle tissue may be a common pathogenic mechanism underlying ALS, SMA, and SBMA pathologies, the expression levels of miR-206, miR-133a, miR-133b, and miR-1 in G93A-SOD1, Δ7SMA, and AR113Q mice were analyzed at presymptomatic, onset, and symptomatic disease stages.

MiR-206 expression levels resulted significantly increased in the symptomatic stages of the three animal models compared to control mice. In particular, in the G93A-SOD1 mice miR-206 upregulation started already from the presymptomatic week 8, and lasted until week 18, suggesting that miR-206 overexpression is an early event prior to evidence of disease symptoms, and continuing during disease course (Fig. 8A, * $p < 0.05$); in Δ7SMA and AR113Q mice, miR-206 upregulation was significant at late disease phase, at day 10 and week 40, respectively (Fig. 8B, 8C, * $p < 0.05$ and ** $p < 0.01$, respectively).

In contrast, miR-133a and miR-133b levels decreased during symptomatic stages in G93A-SOD1 and AR113Q mice compared to control animals (Fig. 8A, 8C). Indeed, miR-133a was downregulated from week 15 to 18, and miR-133b from week 12 (disease onset) to week 18 in G93A-SOD1 (Fig. 8A, * $p < 0.05$, and ** $p < 0.01$), and both miRNAs were significantly decreased at week 40 in AR113Q mice (Fig. 8C, * $p < 0.05$ and ** $p < 0.01$, respectively). In Δ7SMA mice, miR-133a and miR-133b showed an opposite trend, since both myomiR levels were increased at the late stage of day 10, although differences did not reach statistical significance for miR-133b (Fig. 8B, ** $p < 0.01$ for miR-133a).

MiR-1 levels showed a similar expression pattern to that of miR-133a and miR-133b; indeed, it was significantly downregulated in G93A-SOD1 and AR113Q affected mice but increased in Δ7SMA mice. Specifically, lower levels of this miRNA were found in G93A-SOD1 animals already from week 8 until week 18, and in AR113Q mice from week 14 to week 40 (Fig. 8A, 8C, * $p < 0.05$, and ** $p < 0.01$), whereas a significant miR-1 upregulation was observed at day 10 in Δ7SMA mice (** $p < 0.01$).

Of note, in line with data observed in the three MND animal models, significantly increased levels of miR-206 were discovered in denervated (referred as AXO) mice (Fig. 8D, * $p < 0.05$), whereas miR-133a, miR-133b, and miR-1 levels were decreased, as observed in G93A-SOD1 and AR113Q animals (Figure 8D, * $p < 0.05$). Our findings suggest that the dysregulated expression of myomiRs could be significantly associated with the response to muscle wasting which characterizes MNDs.

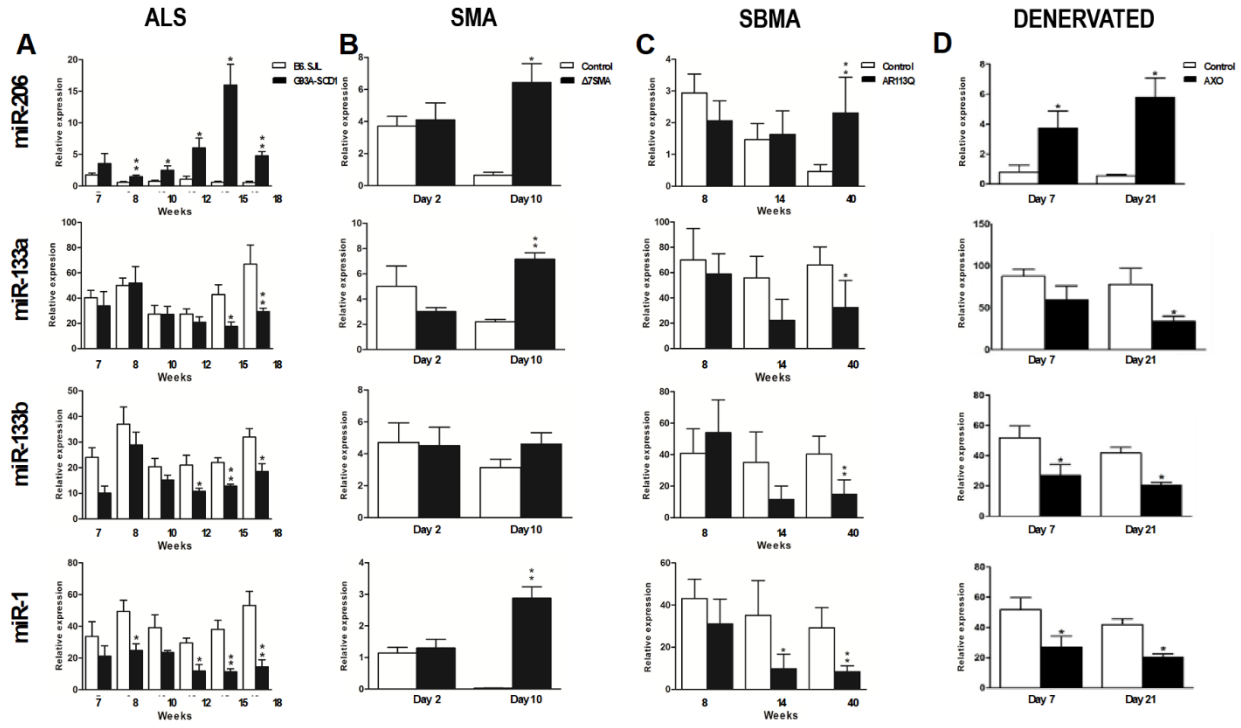


Fig. 8. MyomiRs expression in MND mouse muscle tissue during disease progression. qPCR analysis of myomiRs in (**A**) G93A-SOD1 (black bars), (**B**) Δ7SMA (black bars), (**C**) AR113Q (black bars) (**D**) AXO (black bars) and control mice (white bars) at different disease stages (5 mice per group). Data were normalized to U6 and expressed as $2^{-\Delta Ct}$. Relative expression data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, Mann–Whitney test.

4.2 Altered expression of myomiR target mRNAs in G93A-SOD1, Δ7SMA, and AR113Q muscle tissue during disease progression

Based on the miRWalk 2.0 and miRBase database of predicted and validated miRNA-target interactions (164) and on literature data reporting experimentally validated myomiR targets (99, 106, 109, 165-169), the following myomiR target genes were selected: Pax7, known to be responsible of muscle cell proliferation, Myod1, Myog, and Mef2a, known to be involved in the early and late stages of muscle cell differentiation. These four genes are targets of all the myomiRs considered in this study. Their expression levels were quantified in the same G93A-SOD1, Δ7SMA, and AR113Q mouse muscle samples used for the previous myomiR analysis.
The molecular data revealed no significant changes in *Pax7* mRNA levels in both G93A-SOD1 and Δ7SMA mice compared to controls during disease progression (Fig. 9A, 9B), whereas in AR113Q mice *Pax7* expression levels resulted upregulated in affected mice compared to controls at 14 and 40 weeks (Fig. 9C, * $p < 0.05$)
Regarding *Myod1*, an upregulation of its mRNA levels was evident starting already from disease onset at week 12 until week 18 in G93A-SOD1 mice compared to controls (Fig 9A, * $p < 0.05$ and ** $p < 0.01$ respectively), and, interestingly, *Myod1* upregulation was observed also at the presymptomatic stage of 8 weeks in the AR113Q mice (Fig. 9C, * $p < 0.05$), but significantly decreased at the late stage of the pathology at 40 week (Fig. 9C, * $p < 0.05$). No differences in *Myod1* expression were detected in the Δ7SMA mice compared to healthy littermates (Fig. 9B).
A very similar alteration of *Myog* transcriptional levels was identified. Indeed, an upregulation of its expression was observed in G93A-SOD1 mice (Fig. 9A, ** $p < 0.01$) from 12 to 18 weeks, while in the AR113Q mice this significant increase was present already at week 8 and was confirmed at week

40 (Fig. 9C, * $p < 0.05$). Of note, already at the early stages of the pathology at day 2, Δ7SMA mice showed a strong upregulation of *Myog* levels (Fig. 9B, ** $p < 0.01$).
Lastly, *Mef2a* mRNA levels resulted downregulated in G93A-SOD1 mice compared to controls at 15 and 18 weeks (Fig. 9A, ** $p < 0.01$ and * $p < 0.05$ respectively), as well as in the AR113Q mice at week 40 (Fig. 9C, * $p < 0.05$). No significant difference was detected in the Δ7SMA mice.
In the denervated mice we detected an upregulation of both *Pax7*, and *Myod1* after 21 days of denervation, an upregulation of *Myog* at already form day 7 to 21, and a significant increase of Mef2a at 7 days (Fig. 9D, * $p < 0.05$).

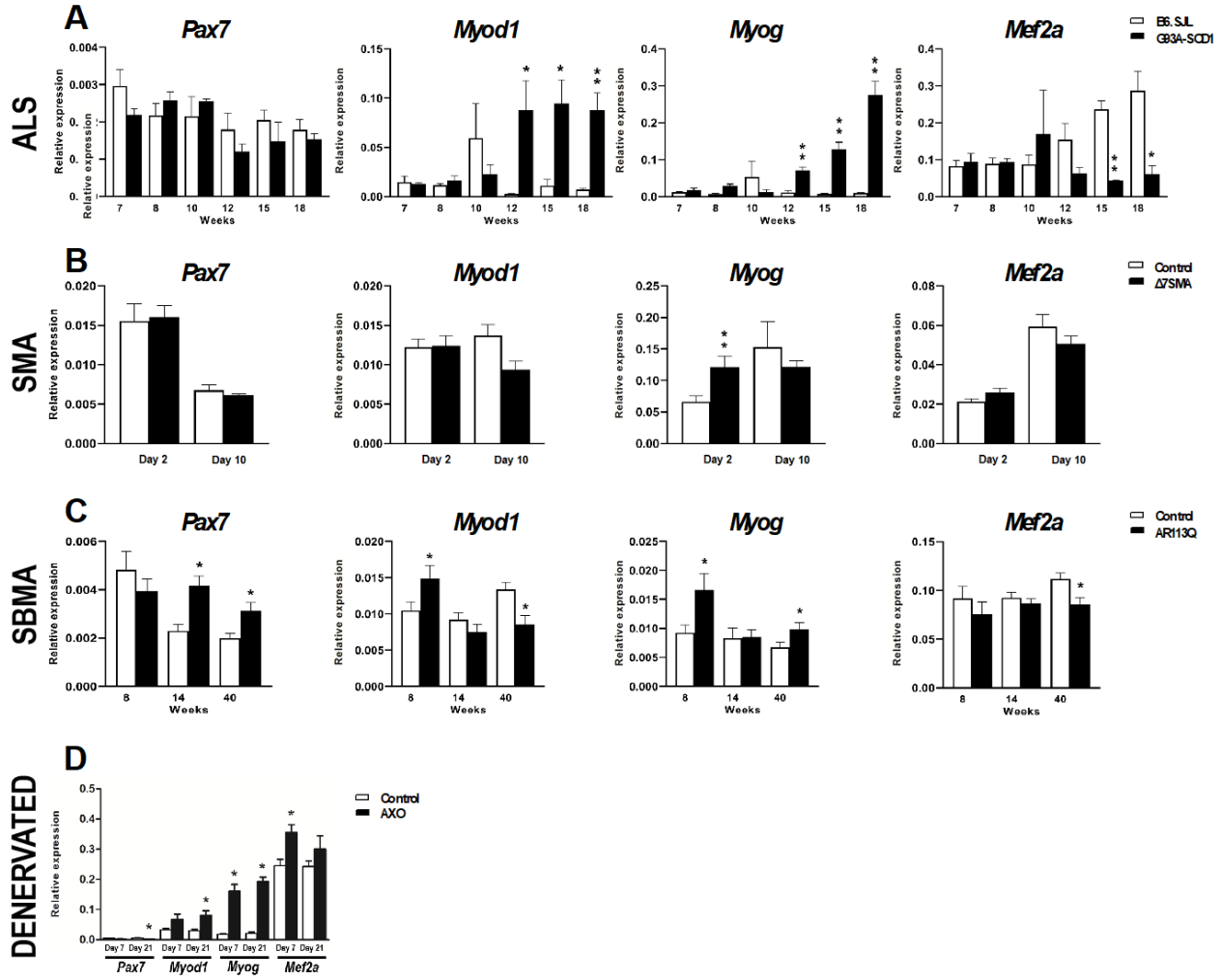


Fig. 9. Target gene expression in MND mouse muscle tissue during disease progression qPCR analysis of myomiR target genes, *Pax7*, *Myod1*, *Myog*, *Mef2a* in (**A**) G93A-SOD1 (black bars), (**B**) Δ7SMA (black bars), (**C**) AR113Q (black bars), (**D**) AXO and control mice (white bars) (5 mice per group). Data were normalized to *Rplp0* mRNA and expressed as $2^{-\Delta Ct}$. Relative expression data are presented as mean ± SEM. * $p < 0.05$, ** $p < 0.01$, Mann–Whitney test.

To check the protein levels of myomiR targets western blot analyses were performed on muscle tissues of the three animal models and controls.
In particular, PAX7 and MEF2A protein expressions were analyzed along with MYOG, chosen since - despite *MyoD* - it acts downstream at the late terminal stages of myogenic differentiation (165, 166).
In line with mRNA expression, in G93A-SOD1 mice no significant alterations were detected in PAX7 protein levels compared to controls, although an increasing trend was highlighted during disease progression (Fig. 10A), and in the AR113Q mice the upregulation of PAX7 was also confirmed at

protein level at the symptomatic stage of week 40 compared to controls (Fig. 10C, * $p < 0.05$). In the Δ7SMA model, we detected a downregulation of PAX7 protein compared to control mice at day 10 (Fig. 10B, * $p < 0.05$).
Interestingly, concordant with transcriptional data, MYOG protein levels were upregulated both in G93A-SOD1 mice starting from week 12 to 18 (Fig. 10A, * $p < 0.05$) and in symptomatic AR113Q mice at 40 weeks compared to controls (Fig. 10C, * $p < 0.05$), whereas no significant alterations were highlighted in Δ7SMA mice.
Regarding MEF2A, we did not detect expression level changes either in G93A-SOD1 or in AR133Q mice, although a slightly decreasing trend was shown, whereas an upregulation of its levels was detected in Δ7SMA mice compared to controls (Fig. 10B, * $p < 0.05$).
A slight reduction of Pax7 mRNA levels was found in denervated mouse model compared to that of controls, along with an upward expression of *Myod1*, *Myog*, and *Mef2a* transcriptional levels, though differences were not confirmed at protein level, with increased trend PAX7 and unchanged levels of MYOG and MEF2A (Fig. 10D).

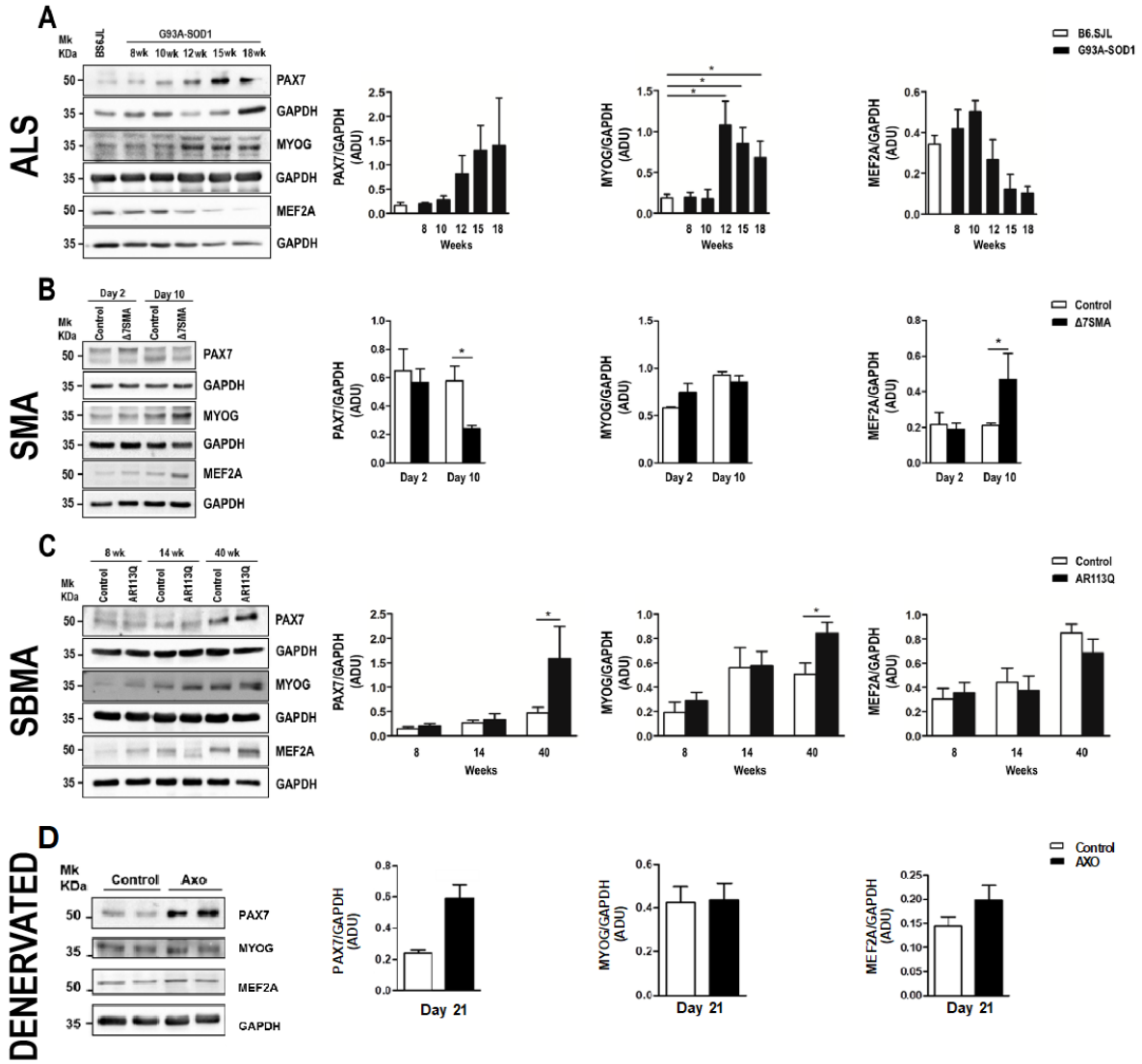


Fig. 10. Protein levels of PAX7, MYOG, and MEF2A in MND mouse muscle tissue during disease progression. Representative western blot analysis of PAX7, MYOG and MEF2A proteins in (**A**) G93A-SOD1 (black bars), (**B**) Δ7SMA (black bars), (**C**) AR113Q (black bars), (**D**) AXO and control mice (white bars) (3 mice per group) with relative densitometric analysis. Density values are reported

as mean ± SEM, corrected for background and normalized to GAPDH control. * $p < 0.05$, Mann-Whitney test.

4.3 MyomiR-target mRNA correlations in muscle tissue of G93A-SOD1, Δ7SMA, and AR113Q mice

We proposed a correlation analysis framework to better understand MND-specific potential relationships between the expression of myomiRs and their putative target genes, throughout disease progression. In particular, since miRNAs can act either as negative or positive regulators of target mRNAs (99, 106, 109, 165-169), we illustrated possible correlations between up and downregulated myomiRs and the up and downregulated target genes in each animal model (Fig. 11A, 11B, 11C), taking into account both inverse and direct correlations.
Based on the mean of quantitative real-time PCR values, Spearman's correlation analysis was performed (Table 2), considering coefficient r lower than −0.5 or higher than +0.5 as good inverse or direct correlation, respectively.
The following myomiR-mRNA pair correlations were identified as the most conspicuous and significant: miR-206/*Pax7* and miR-206/*Myod1* in G93A-SOD1 mice (Fig. 12A); miR-133a/*Pax7*, miR-1/*Pax7*, and miR-1/*Mef2a* in Δ7SMA mice (Fig. 12B); miR-133a/*Myod1*, miR-133b/*Myod1*, miR-133b/*Myog*, miR-1/*Pax7*, miR-1/*Myod1*, and miR-1/*Myog* in AR113Q mice (Fig. 12C). In denervated mice we did not observe significant negative or positive correlations between each myomiR and the putative targets ($p > 0.05$).

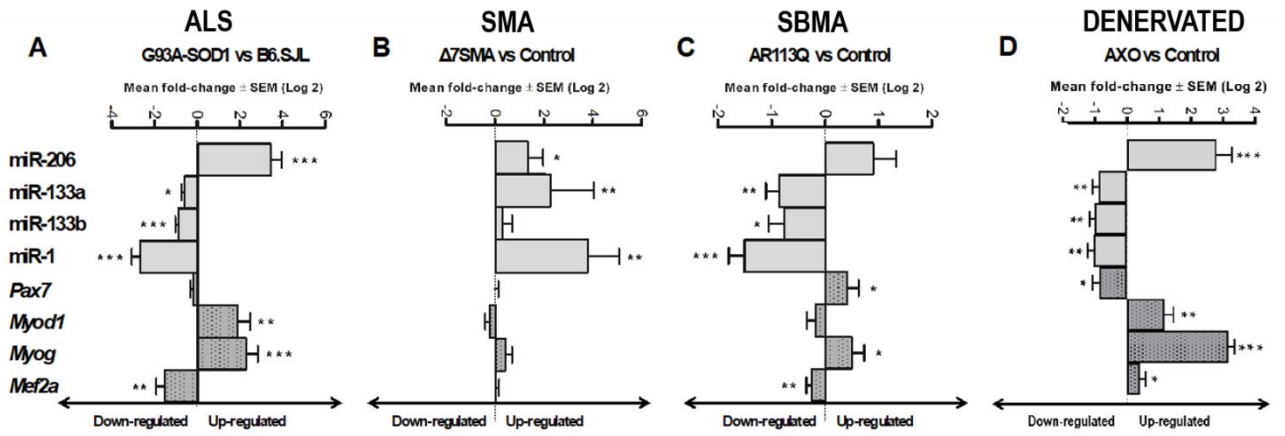


Fig. 11. MyomiR and target gene expression in MND mouse model muscle tissue. Data are presented as mean ± SEM of $\log_2$ of fold changes of $2^{-\Delta Ct}$ expression of myomiRs (grey bars) and target mRNAs (grey dotted bars) obtained at different disease stages in (**A**) G93A-SOD1, (**B**) Δ7SMA, (**C**) AR113Q, and (**D**) AXO mice compared to controls. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Mann–Whitney test.

myomiRs	Target Genes	G93A-SOD1	Δ7SMA	AR113Q
miR-206	*Pax7*	**r_s= -0.613; p= 0.006**	r_s < + 0.5	r_s < + 0.5
	Myod1	**r_s = 0.587; p= 0.010**	r_s < + 0.5	r_s < + 0.5
	Myog	r_s < + 0.5	r_s < + 0.5	r_s < + 0.5
	Mef2a	r_s < + 0.5	r_s < + 0.5	r_s < + 0.5
miR-133a	*Pax7*	r_s < + 0.5	**r_s = −0.672; p = 0.039**	r_s < + 0.5
	Myod1	r_s < + 0.5	r_s < + 0.5	**r_s = 0.628; p = 0.012**
	Myog	r_s < + 0.5	r_s < + 0.5	r_s < + 0.5
	Mef2a	r_s > - 0.5	r_s > + 0.5	r_s < + 0.5
miR-133b	*Pax7*	r_s < + 0.5	r_s < + 0.5	r_s < + 0.5
	Myod1	r_s < + 0.5	r_s < + 0.5	**r_s = 0.803; p = 0.0003**
	Myog	r_s < + 0.5	r_s < + 0.5	**r_s = 0.775; p = 0.0007**
	Mef2a	r_s < + 0.5	r_s < + 0.5	r_s < + 0.5
miR-1	*Pax7*	r_s < + 0.5	**r_s = −0.685; p= 0.035**	**r_s = 0.620; p = 0.023**
	Myod1	r_s < + 0.5	r_s < + 0.5	**r_s = 0.573; p = 0.002**
	Myog	r_s < + 0.5	r_s < + 0.5	**r_s = 0.714; p = 0.006**
	Mef2a	r_s < + 0.5	**r_s = 0.676; p = 0.031**	r_s < + 0.5

Table 2. Results of Spearman myomiR-mRNA target correlations in G93A-SOD1, Δ7SMA and AR113Q mouse muscle. Analyses assessed positive or negative correlations between the expression levels of each myomiR and the mRNA levels of the target genes in G93A-SOD1, Δ7SMA and AR113Q mice at the different disease stages.

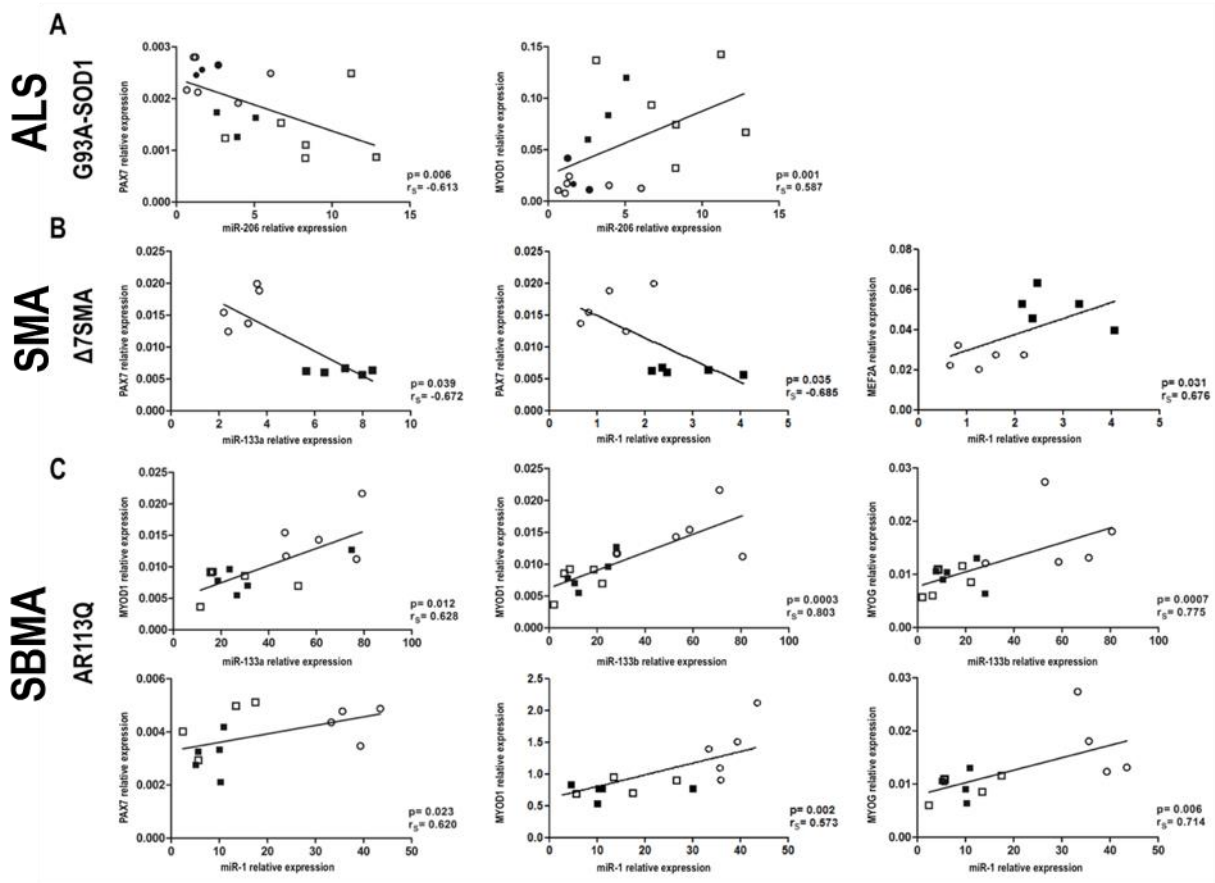


Fig. 12. MyomiR-target mRNA correlation analysis in MND mouse muscle tissue during disease progression. Negative and positive correlations ($p < 0.05$) estimated by Spearman's correlation test between myomiR and mRNA levels of *Pax7*, *Myod1*, *Myog*, and *Mef2a* in muscle tissue of: (**A**) G93A-SOD1 at weeks 7 (●), 8 (○), 10 (●), 12 (□), 15 (■), and 18 (■); (**B**) Δ7SMA at day 2 (○) and 10 (■); (**C**) AR113Q at weeks 8 (○), 14 (□), and 40 (■).

4.4 Alterations of G93A-SOD1, Δ7SMA, and AR113Q muscle architecture at late disease stages

To evaluate muscle atrophy, we performed histological analysis of gastrocnemius muscle specimens in the three MND animal models at late disease stage when the animals showed the most striking molecular changes. Morphological analysis by hematoxylin/eosin staining demonstrated that skeletal muscles from G93A-SOD1 (Fig. 13A), Δ7SMA (Fig. 13B), AR113Q (Fig. 13C), and AXO (Fig. 13D) mice were characterized by the presence of smaller fibers, larger connective endomysial spaces, and few fibers with centrally located nuclei in relation to age-matched controls. As we already showed in G93A-SOD1 animal model (34) and in agreement with a previous report (163), a gradually worsening in terms of muscle disorganization was evident at late disease stage. In G93A-SOD1, we confirmed a significant reduction of muscle fiber diameters compared to controls (Fig. 13A, * $p < 0.05$).
A similar reduction in muscle fiber diameter was observed in Δ7SMA mice compared to controls at day 10 (Fig. 13B); in this mouse model muscle fiber diameter was generally smaller compared to the other MND animal models due to the lower age at the time of analysis.
Similar results were observed in AR113Q mice compared to control at week 40 (Fig. 13C). In line with data observed in the three MND animal models, we detected similar morphological changes also in the denervation mouse model (Fig. 13D).

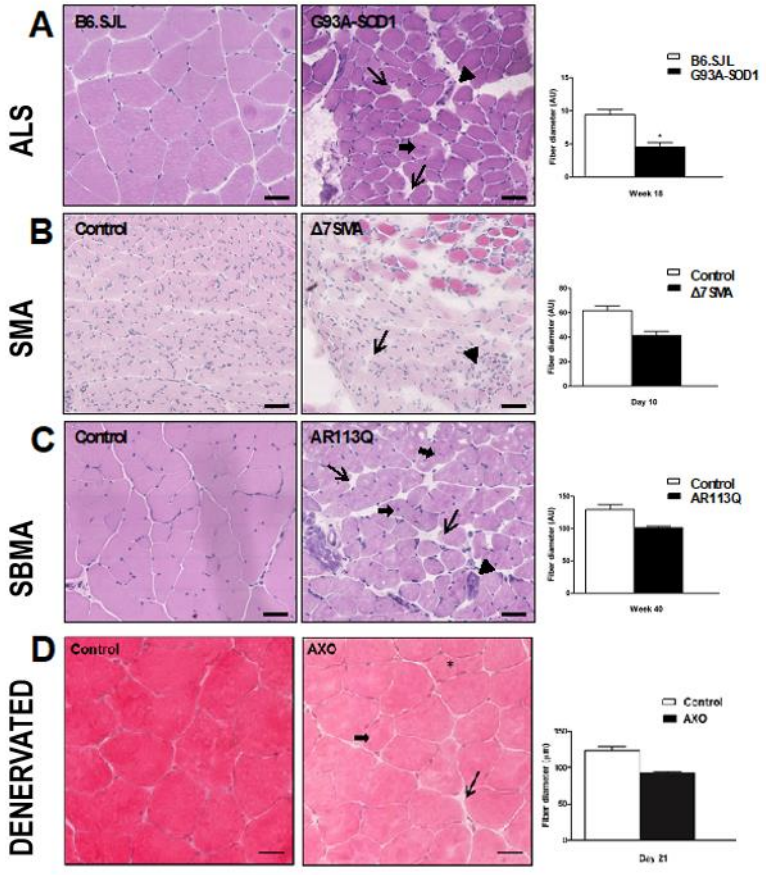


Fig. 13. Histological analysis of gastrocnemius muscle fiber diameter in MND mouse model muscle tissue. Representative transversal skeletal muscle sections stained with hematoxylin/eosin in (**A**) G93A-SOD1 mice, (**B**) Δ7SMA mice, (**C**) AR113Q mice, and (**D**) AXO mice (right columns) at late disease stages (week 18, day 10, and week 40, respectively), and relative age-matched controls (left columns). Long arrows indicate enlarged endomysial spaces; arrowheads indicate degenerating cells with apoptotic fragmented nuclei; short arrows indicate fibers with centrally located nuclei, common during muscle regeneration. Magnification 40X. Scale bar = 50μm. Graphs on the right,

measurement of fiber diameter in gastrocnemius muscle of G93A-SOD1 at week 18 (black bars), Δ7SMA mice at day 10 (black bars), AR113Q mice at week 40 (black bars), and relative age matched control mice (white bars). Each histogram represents the mean diameter (μm) ± SEM of muscle fibers measured in three muscle sections per mouse from three mice in each group. * $p < 0.05$. Mann–Whitney test

4.5 Altered myomiR expression in serum samples of ALS, SMA, and SBMA patients

To evaluate the potential of miR-206, miR-133a, miR-133b and miR-1 as noninvasive biomarkers for MNDs, we assessed their expression levels in serum specimens of ALS patients carrying SOD1 mutation, pediatric SMA, SBMA patients, and sex- and age-matched control subjects (Table 3).

Patients	SOD1-mutated ALS	SMA	SBMA	Anti-NMDA receptor encephalitis	Healthy controls
Number of patients	n=14	n=23	n=10	n=19	n=17
Sex (F/M)	7/7	13/10	0/10	12/7	8/9
Age at onset (Years, mean ± SD)	50 ± 12.2	6.86 ± 3.33	56.3 ± 5.92	13.13 ± 5.62	48.64 ± 11.33
Disease-related information	SOD1 mutation sporadic/familial (8/6)	SMN1 deletion SMA type II/III (17/6)	CAG expansion 38-62 repeats	Autoimmune encephalitis	-

Table 3. Summary of the main features of ALS, SMA and SBMA patients and controls included in the study

We found a significant increase of miR-206 levels in ALS and SMA patients' sera, while in SBMA serum samples miR-206 expression showed the same upward trend, but the difference between patients and healthy subjects was not significant (Fig. 14A). On the other hand, we did not find significant differences in miR-133a, miR-133b, and miR-1 expression in ALS, SMA, and SBMA patients compared to controls (Fig. 14B, 14C, 14D).
To determine the diagnostic value of miR-206 as serum biomarker in ALS and SMA patients we evaluated its sensitivity and specificity by ROC curve analysis which supported a possible role for circulating miR-206 as disease biomarker (Fig. 15A, 15B).

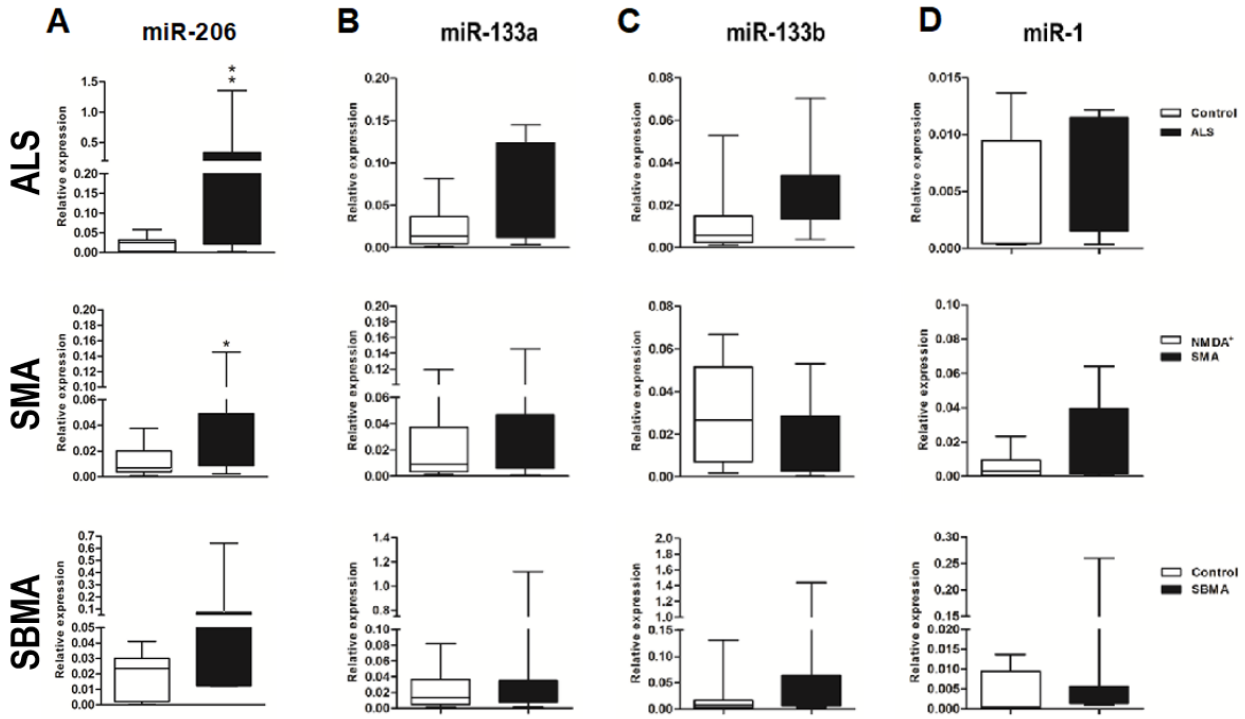
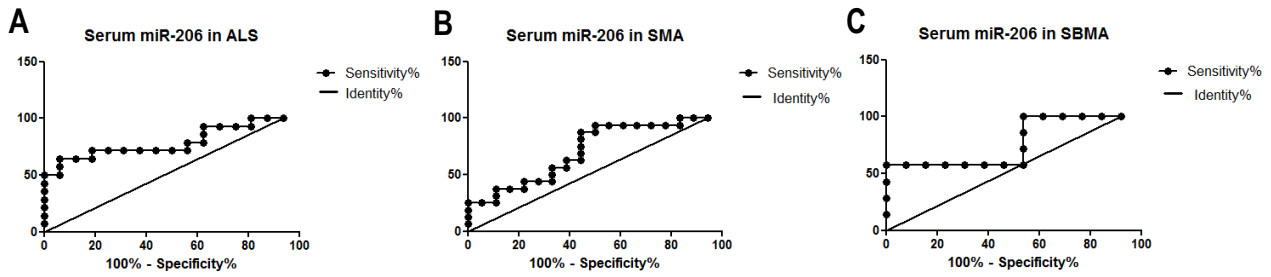


Fig. 14. MyomiR expression in serum samples of ALS, SMA, and SBMA patients. qPCR analysis of myomiRs in serum of ALS, pediatric SMA, and SBMA patients (black bars), healthy controls (white bars) and patients with anti-NMDA receptor encephalitis (white bars in the SMA graph) as controls for pediatric SMA patients. Data were normalized to miR-16 and expressed as $2^{-\Delta Ct}$. Relative expression data are presented as mean ± SEM.* $p < 0.05$, ** $p < 0.01$, Mann–Whitney test.



Area	0.7902
Std. Error	0.08764
95% confidence interval	0.6184 to 0.9620
P value	0.006914

Area	0.7118
Std. Error	0.08919
95% confidence interval	0.5370 to 0.8867
P value	0.03537

Area	0.7692
Std. Error	0.1197
95% confidence interval	0.5346 to 1.004
P value	0.05226

Fig. 15. Receiver operating characteristic (ROC) curves assessed the sensitivity and specificity of miR-206 in serum as biomarker for ALS, SMA, and SBMA.

5. DISCUSSION

Growing evidence is shedding lights on the crucial role of skeletal muscle in MNDs, suggesting that not only at the NMJ but also at the skeletal muscle level, intrinsic pathogenic mechanisms may lead to an axon-somatic backpropagation able to trigger MN dying back (34, 130, 163, 171).The understanding of the molecular etiology underlying MND muscle pathology is fundamental for the identification of both tailored therapeutic interventions and the potential of skeletal muscle as novel treatment target tissue. In particular, the discovery of common pathogenetic features and coordinated molecular activities shared by similar disease conditions such as MNDs can provide groundbreaking insight for complex diseases such ALS and speed up the development of effective cure.

In this study we analyzed the expression of muscle-specific miRNAs, myomiRs, and their putative target genes in the skeletal muscle tissue of MND animal model, including G93A-SOD1, Δ7SMA and AR113Q mice, to understand the complex molecular pathways underlying atrophy in MNDs. Next, considering the lack of ultimate effective biomarkers for MNDs and the promising characteristics of myomiRs as suitable biomarkers, we analyzed their expression in serum samples from MND patients.

The most conspicuous result of our analyses is the identification of a common miR-206 upregulation in all the three animal models, at the late disease stages, as well as in the denervated mice, highlighting a muscle-specific dysregulated mechanism shared by MNDs, in response to disease progression. This information led to hypothesize that in an atrophic environment, miR-206 overexpression is a final attempt to counteract muscle degeneration and the subsequent motoneuron dying back, being miR-206 a crucial player for both muscle and NMJ regeneration (120, 122). Interestingly, miR-206 resulted upregulated already at the pre-symptomatic stage of 8 weeks in the G93A-SOD1 mice. In the ALS animals several pathogenic muscle changes occur prior to clinical sign and MN degeneration (34). Therefore, the early activation of miR-206 could be an attempt to prevent muscle wasting before disease worsening. A significant direct correlation linking miR-206 and *Myod1* was found in G93A-SOD1 mice. Indeed, a concordant upregulation of *Myod1* mRNA was detected from disease onset until 18 weeks, confirming a positive feedback loop (99), overall resulting in an activation attempt of muscle differentiation in response to skeletal muscle denervation. In this scenario, *Myog* mRNA and protein upregulation in G93A-SOD1 from week 12 onwards strengthens our hypothesis regarding a strong attempt to counteract the progressive atrophy by enhancing cell differentiation rate. On the other hand, according to literature (167) a negative correlation between miR-206 and *Pax7* was identified in ALS animals, resulting in muscle proliferation inhibition (167). However, Pax7 mRNA and protein levels were not altered in ALS mice. Recent findings reported that miRNA can show a variable affinity for their mRNA targets, and some mRNAs are more responsive to miRNA perturbation than others (172). Moreover, the accepted model of miRNA targeting that consists of an interplay between AGO, miRNA, and mRNA target, is being revised by taking a much broader context of >1,500 co-regulating RNA binding proteins (RBPs) into additional consideration (173). Therefore, we can speculate that being both Myod and Pax7 targets of miR-206, no changes in the Pax7 levels could be the result of either a higher affinity of miR-206 towards *Myod1* regulation in this specific stage of the disease, or a binding efficacy perturbation between miR-206 and Pax7 caused by additional players (i.e. RBPs).

For the first time we analyzed the expression levels of myomiRs in the SBMA animal model, also demonstrating an up-regulation of miR-206 at the symptomatic disease stage, compared to controls.

In agreement with previous works (130, 160), an upregulation of miR-206 was found in Δ7SMAmice compared to controls.These findings unequivocally disclose that miR-206 up-regulation is a common pathogenic feature associated with muscle atrophy occurring throughout MND course, although further investigation is needed to deeply characterize functional miR-206/target gene interactions in relation to specific disease pathogenesis and progression.

A downregulation of miR-133a was found in the symptomatic stages in G93A-SOD1 and AR113Q and denervated mice, suggesting a common alteration of the proliferation pathway. In particular, in the ALS context, this data reflects what has been observed in ALS patient's biopsies (128) and is in line with the abovementioned described regenerative attempt towards myoblast differentiation rather than proliferation. In the SBMA mouse miR-133a downregulation directly correlated with *Myod1* expression, that resulted significantly reduced in the symptomatic stage. This mechanism supports evidence of the positive feedback loop linking Myod and miR-133 (109), triggering muscle proliferation impairment. However, as mentioned before, miR-133a and -133b play a dual role modulating both muscle proliferation and differentiation (109, 110). In particular, the Myod-miR-133a axis has been described to be indispensable for myogenic differentiation (113), corroborating the hypothesis that muscle differentiation is compromised in SBMA mice. In Δ7SMA animals we identified an upregulation of miR-133a at late disease stage, that inversely correlated with Pax7. Pax7 protein levels were in fact downregulated at day 10, at this stage suggesting an inhibition of cell proliferation able to prevent improvements of muscle condition. Interestingly, an upregulation of miR-133a was observed in SMA patients and the decrease of its expression levels correlated with a positive motor function response upon nusinersen treatment (154).

In line with miR-133a, miR-133b resulted downregulated from disease onset in the G93A-SOD1 mice and the symptomatic stages in the AR113Q animals, but no alteration were detected in Δ7SMA mice. MiR-206 and miR-133b genes are clustered; however, it has been demonstrated that they may be transcribed either as a single bicistronic RNA or, under specific conditions, from separate promoters giving rise to two primary transcripts (112) for myogenic conversion in vitro and in denervation in vivo (120). This evidence explains our results showing opposite expression trends of these myomiRs, in line with previous studies (153). In the G93A-SOD1 animal miR-133b downregulation once more indicated defects in the proliferation pathway, which is not able to support the differentiation attempts to counteract muscle atrophy.

In the AR113Q mice a direct correlation between miR-133b and *Myod1* was found, confirming findings in C2C12 cell regarding Myod-dependent modulation of the miR-206/133b locus (123). Indeed, miR-133b/*Myod* downregulation at symptomatic stage corroborates the idea of a proliferation impairment in SBMA, along with a differentiation disruption, as also confirmed by *Mef2a* significant decrease.

MiR-1 resulted downregulated in G93A-SOD1 and AR113Q and, interestingly, miR-1 decrease was observed in ALS patient muscle biopsies (128). Based on evidence showing that miR-1/miR-133a bicistronic cluster is regulated by Mef2 in a positive feedback loop (109) we speculate that miR-1 and Mef2 concomitant reductions in ALS and SBMA underly defects in muscle differentiation in the pathologies. As miR-133a, miR-1 expression was increased in symptomatic Δ7SMA muscle, confirming a specific muscle pathological signature. Indeed, unlike adult-onset ALS and SBMA, SMA is an aggressive developmental disease, therefore alternative molecular pathways may lead to muscular damage up to atrophy. It has been shown that sensitivity to SMN reduction is highest in younger Δ7SMA animals and results in a severe form of SMA (174). SMN deficiency in satellite cells or myoblasts impaired myofiber growth and regeneration (175). Indeed, little evidence of reinnervation in severe SMA human patients or mice were shown, whereas milder SMA patients has robust reinnervation. In this view, the increase in myomiR expression in severe SMA mice at late disease stage could represent a failed attempt to counteract atrophy. As a matter of fact, we previously demonstrated an upregulation of myomiRs in SMA patients' serum that decreased upon nusinersen treatment (154). Our studies might suggest that reductions of myomiR expression levels could reflect a skeletal muscle stabilization and improvement. Lastly, we can speculate that the aggressive disease progression rate and short lifespan of SMA mice may dampen the identification of miRNA molecular pattern similar to that observed in ALS and SBMA mice.

Hence, a potential regenerative response in muscle tissue of MND animals may be triggered during disease progression, and the dysregulation of specific myomiR/target gene pairs may account for muscle impairment and inefficient repair mechanisms in MNDs. Indeed, we previously showed (34)

a significant increase in hypotrophic fibers, mainly in ALS and SBMA animal model, congruent with clinical worsening (34, 163). Some of these fibers present morphological signs of degeneration, and others are smaller as possible results of a defective regenerative attempt, in line with the presence of very few regenerating muscle cells (34, 176). Although some hypotrophic fibers were detected also in SMA skeletal muscle, general fiber morphology was mostly preserved in Δ7SMA mice. Here, as mentioned, a defective activity of the satellite cells may be responsible for the failure to mature to muscle fibers (177), in line with the dysregulated myomiR/target gene patterns that we detected.
Since miRNAs are stable in body fluids and may reflect distinct pathophysiological states, they represent promising biomarkers for MNDs. These molecules can be released into the circulation by pathological affected tissues and display remarkable stability in body fluids. Indeed, in a previous study it was demonstrated that miR-206 was up-regulated in skeletal muscle and plasma of G93A-SOD1 mice and in serum of ALS patients, suggesting that this molecule can reflect the pathological state of muscle in the body fluids (153). This phenomenon was observed also in SMA mice and patients, where an up-regulation of miR-206 was detected in skeletal muscle tissue and serum (160). Biomarkers need to be minimally invasive to be feasibly detected in clinical practice. Thus, we then investigated the selected myomiRs in the corresponding MND patients' serum samples. We found increased levels of miR-206 in ALS, as previously reported (126, 178), and notably, also in SMA sera, along with a similar trend in SBMA patients. Thus, similar molecular regulatory mechanisms are triggered in response to pathological processes occurring in muscles, both in animal model and in humans, and miR-206 levels in serum may reflect disease status and a compensatory response to cope with muscle atrophy (179). Further investigations increasing the sample size may be required to strengthen our results. However, our miR-206 sensitivity and specificity data supported its possible usefulness for monitoring disease progression. In this view, a serum decreased expression of myomiRs after therapeutic treatment might be the result of an improvement of muscle conditions and performance (154). Likewise, in the field of ALS, Pegoraro and colleagues recently found a significant decrease in serum levels of miR-206 after physical training in ALS patients associated to stabilization of skeletal muscle and neuromuscular junction (180).
Overall, we identified a common miR-206 signature underlying ALS, SMA and SBMA muscle impairment. Defects in muscle regeneration rely on altered myomiR and target gene expression involved in both muscle proliferation and differentiation. MyomiR are functionally redundant (123) and deletion of one myomiR may be compensated by others targeting the same genes (151). However, the combinatory decrease of miR-133a, miR-133b and miR-1 suggests a noteworthy disrupted mechanism not able to sustain the regenerative attempt of miR-206. Further investigation is needed to deeply characterize functional myomiR/target gene interactions in relation to specific disease pathogenesis and progression. A therapeutic strategy based on myomiR mimic delivery into skeletal muscle could represent a novel therapeutic strategy for ALS and SBMA. Moreover, to strengthen the therapeutic delivery and avoid degradation, liposome- or nanoparticle-based strategies for myomiR packaging could be taken into account. In addition, our data highlight the potential of myomiRs as noninvasive biomarkers in ALS, SMA, and SBMA.

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