Noncoding RNAs in Duchenne and Becker muscular dystrophies: role in pathogenesis and future prognostic and therapeutic perspectives

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Abstract

Noncoding RNAs (ncRNAs), such as miRNAs and long noncoding RNAs, are key regulators of gene expression at the post-transcriptional level and represent promising therapeutic targets and biomarkers for several human diseases, including Duchenne and Becker muscular dystrophies (DMD/BMD). A role for ncRNAs in the pathogenesis of muscular dystrophies has been suggested, even if it is still incompletely understood. Here, we discuss current progress leading towards the clinical utility of ncRNAs for DMD/BMD. Long and short noncoding RNAs are differentially expressed in DMD/BMD and have a mechanism of action via targeting mRNAs. A subset of muscle-enriched miRNAs, the so-called myomiRs (miR-1, miR-133, and miR-206), are increased in the serum of patients with DMD and in dystrophin-defective animal models. Interestingly, myomiRs might be used as biomarkers, given that their levels can be corrected after dystrophin restoration in dystrophic mice. Remarkably, further evidence demonstrates that ncRNAs also play a role in dystrophin expression; thus, their modulations might represent a potential therapeutic strategy with the aim of upregulating the dystrophin protein in combination with other oligonucleotides/gene therapy approaches.

Keywords Duchenne muscular dystrophy · Becker muscular dystrophy · lncRNA · miRNA · Biomarkers · Antisense oligonucleotides

Introduction

Duchenne muscular dystrophy (DMD) is one of the most common neuromuscular disorders in childhood, involving around 1 in 3500 male births [1] with an incidence from 10.71 to 27.78 per 100.000 males [2]. It is an X-linked disease, usually caused by out-of-frame mutations in the dystrophin gene *DMD*, which leads to the absence of protein expression. On the contrary, mutations retaining the reading frame are generally related to partial residual expression of dystrophin and a milder phenotype called Becker Muscular Dystrophy (BMD) [3].

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Dystrophin is a cytoplasmatic protein that plays a major structural role in skeletal muscles, linking the cytoskeleton to the extracellular matrix via a complex (dystrophin-associated protein complex or DAPC) formed with dystroglycans, sarcoglycans, sarcospan, dystrobrevins and syntrophin [4]. The disruption of this structure, especially the linking with actin and beta-dystroglycan, destabilizes the sarcolemma during the muscle contraction [5]. Membrane instability allows the entrance of calcium ions with subsequent oxidative stress, activation of Ca^{2+}-dependent calpain and protein degradation. Activation of complement and infl
causes necrosis and generation of pro-fibrotic factors. While muscle regeneration initially increases to replace muscle damage, over time, muscle regeneration fails and fibro-fatty substitution takes place, impairing the muscle function [6]. In fact, the prolonged exposure to an adverse environment prevents myogenesis, despite satellite cells retaining regenerative capacity [7].

Moreover, dystrophin has an important scaffolding role, participating in the cell signaling pathways [8] and enabling the correct localization of nitric oxide synthetase (nNOS) [9]. Other components of DAPC are also involved

in signaling. For instance, beta-dystroglycan is involved in MAPK pathway, dystroglycans are cell surface receptors for protein of the extracellular matrix, syntrophin organizes several signaling proteins and interacts with ion channels and G-protein-related pathways [10]. The absence of this important scaffolding role can result in disrupted expression of several interrelating molecules.

DMD generally starts in early childhood with proximal muscle weakness leading to loss of ambulation at approximately 12 years of age. Diagnosis is usually achieved at 3 years of life due to motor delay, sural hypertrophy, falls or increased CK levels. When 6-year-old, the patients develop progressive difficulties in running, climbing stairs and rising from floor. The muscles most affected by fibrofatty degeneration are psoas, gluteus and quadriceps, followed by proximal muscles of upper limbs [11–13]. Cardiac involvement, starting from 10 years of age, leads to dilated cardiomyopathy and heart failure [14]. Death occurs within the third decade of life due to heart or respiratory failure [15]. On the other hand, BMD is a milder disease, although more heterogeneous, characterized by muscle weakness and atrophy with juvenile onset and sural hypertrophy. Loss of ambulation may occur at variable ages, even after 60 years of age [16]. Cardiomyopathy is less frequent than DMD but represents one of the most frequent causes of death in BMD subjects and it is not related to the level of skeletal muscle impairment [17].

To date, no definitive cure for dystrophinopathies is available, but novel therapeutic strategies have been attempted [18]. Recently, three novel therapies have been approved by the FDA (Eteplirsen and Golodirsen) and EMA (Ataluren). Exon skipping, mediated by oligonucleotides, allows for the production of a shorter but functional mRNA rescuing dystrophin expression and shifting from DMD to BMD-like phenotype. Eteplirsen is a phosphorodiamidate morpholino oligomer able to restore the dystrophin frame of lecture in subjects carrying a deletion amendable of skipping of exon 51 (approximately 14% of DMD patients) [19, 20]. Similarly, Golodirsen induces skipping of exon 53 [21]. Instead, Ataluren is a small molecule able to bypass dystrophin stop codon mutations by a read-through mechanism, allowing the expression of the protein [22].

Other strategies have been proposed to modify the course of the disease. For example, gene therapy delivers smaller forms of *DYS* with adeno-associated viral vector (AAV) and has been tested in clinical trials [23]. Genome editing through CRISPR–Cas9 technology has been attempted in animal models [24]. Epigenetic mechanisms can also be considered a therapeutic target. Post-transcriptional regulation could be implicated in the pathogenesis of the disease, as indicated by the extremely variable dystrophin expression (8–63%) detected by analyzing BMD patients carrying the same mutation (Δ45-47) and comparable levels of dystrophin mRNA [25]. Moreover, several compounds target the pathological changes related to the loss of dystrophin and include corticosteroids, other anti-inflammatory drugs, antioxidants as Idebenone, vasodilators as Tadalafi and myostatin inhibitors [26].

In this context, non-coding RNAs (ncRNAs) have emerged as relevant molecules in the pathogenesis of several human disorders. Non-coding RNAs are not translated into proteins and originate from parts of codifying genes or are transcribed as independent genes. They can be classified according to their function as RNAs involved in translation (transfer RNA—tRNA, ribosomal RNA—rRNA), splicing (small nuclear RNA—snRNA), modifi of small RNAs (small nucleolar RNA—snoRNA), regulation of gene expression (piwi-associated RNA—piRNA, endogenous short-interfering RNA, microRNA—miRNA). Moreover, ultraconserved regions (T-UCR), large intergenic noncoding RNA (lincRNA), long noncoding RNA (lncRNAsmall interfering RNAs (siRNAs) and circular RNA have been described [27–29]. ncRNAs are involved in many different cellular pathways, including development and differentiation, tissue homeostasis, signal transduction and cell proliferation. They may also be identified in several biological fluids, like blood or urine, inside exosomes or linked to lipoproteins or freely circulating, thus representing extracellular biomarkers [27].

The most studied ncRNAs are represented by microRNAs, small RNA sequences (approximately 22 nucleotides) acting as post-transcriptional modifi MiRNAs repress target gene expression by binding the 3′ untranscribed region (3′UTR) of the corresponding mRNA with subsequent degradation or translation inhibition [31]. In certain conditions, they also improve gene expression, stabilizing the mRNA and facilitating its translation [32].

Long noncoding RNAs (lncRNAs) are molecules longer than 200 nucleotides, localized both in the nucleus and cytoplasm, encoded proximally to promoters of coding genes and poorly conserved across species [33]. They are implicated in diff ent cellular pathways, such as chromatin remodeling through histone methylation or acetylation or acting as a scaff for other enzymes, transcriptional activation or interference, cell traffi king, protein relocalization and phosphorylation or ubiquitination, mRNA processing and stability, splicing regulation and post-transcriptional and post-translational processes [34, 35]. LncRNAs can also be miRNAs precursors. Moreover, several types of noncoding RNAs, including lncRNAs and circular RNAs, are able to bind miRNAs, acting as ‘sponges’ and sequestering them from their targets [35–37].

Non-coding RNAs have been recently studied in dystrophinopathies to evaluate their role in the pathogenesis of the disease, as dystrophin expression modulators, as biomarkers and as possible therapeutic targets. This review will focus

on the impact of miRNAs and lncRNAs on these aspects (Table 1).

Modulators of dystrophin expression

Dystrophin expression in skeletal muscle can be directly modulated by specific miRNAs

Fiorillo et al. analyzed muscle specimens of 10 BMD patients carrying a *DMD* deletion 45–47. Subjects were divided into two groups, low-BMD and high-BMD, according to dystrophin levels by Western blot analysis (cut-off 20%). Since protein expression was not directly related to dystrophin mRNA levels, muscle expression of specifi miRNAs binding dystrophin 3′UTR was studied. Selected miRNA levels were significantly overexpressed in low-BMD (miR-146b-5p, miR-382, miR-410, miR-758, miR-214, miR-494, miR-223, miR-146a, miR-195, miR-374a, miR-103, miR-320a, let-7d, let-7a) and high-BMD subjects (miR-410, miR-758, miR-214, miR-223, miR-494). An inverse correlation between miRNA levels and protein expression was detected. Additionally, miR-31 was upregulated in DMD patients [25].

Likewise, animal models of dystrophinopathies showed an upregulation of miR-146b, miR-146a, miR-223 and miR-382 as detected in the vastus lateralis (50-fold, 3-fold, 8-fold) and sartorius (25-fold, 1.3-fold, 2.5-fold, 4-fold, respectively) of the Golden Retriever muscle dystrophy (GRMD) model. Similar upregulations were detected in the gastrocnemius muscle of the *mdx* mouse. Moreover, a direct correlation between age and miRNA levels was found in GRMD (miR-146b, miR-146a, miR-223) and *mdx* (miR-223, miR-31) models in comparison to wild-type controls [25].

Among the 14 miRNAs upregulated in the low-BMD group, Fiorillo identified 7 miRNAs inhibiting (miR-146b, miR-31, miR-374 and, to a lesser extent, miR-146a, miR-223, miR-320a, miR-382) and 2 miRNAs enhancing dystrophin expression (miR-195, miR-758) [25]. Only limited knowledge of the actual muscular function of these miRNAs is available.

Furthermore, 14 lncRNAs originate from the DMD locus, most of them from intron regions close to dystrophin promoters or isoform-specifi fi t exons, since they are involved in the modulation of protein expression through targeting of their promoters. These transcripts localize to the nucleus and are expressed in at least one among skeletal muscle, heart, or brain. Transcription of these lncRNAs seems to be related to dystrophin and primed by myogenic diff entiation induced by MyoD. Their overexpression in human muscles and neuronal cells causes downregulation of brain and muscle dystrophin full-length isoforms (Dp427b and Dp427m) targeting the promoter; instead, Dp427p and Dp71 transcription are barely affected. A negative correlation between lncRNAs and full-length DMD isoform levels has been found in dystrophinopathy female carriers [38].

Pathogenesis of dystrophinopathies

In addition to modulating dystrophin expression, miRNAs are involved in signaling pathways, development and cellular phenotype and regulation of myogenic proliferation and fibrosis.

Greco et al. suggested a classification of miRNAs involved in muscular pathways into miRNAs associated with 1) inflammation (miR-222, miR-223), expressed in response to muscle fiber damage; 2) degeneration (miR-1, miR-29c, miR-135a), reduced in the *mdx* model or DMD patients or after ischemia and linked to myofi loss and fi osis; 3) regeneration (miR-31, miR-34c, miR-206, miR-335, miR-449, miR-494), increased in the *mdx* model, DMD patients and in response to induced ischemia [39].

Infl Fiorillo et al. further evaluated the role of miRNAs in the infl related to the dystrophic process. They treated *mdx* myotubes with TNF-alpha and detected an increase in miR-146a and miR-223 levels. Since steroid therapy is one of the gold standards in DMD management, prednisolone was administered to reverse miRNA changes. Anti-infl ory drugs also decrease miR-146b and miR-382 levels. Therefore, they speculated that TNF-alpha-related inflammation might activate the NFkB pathway in muscle fi inducing miR-146a and miR-223 expression and inhibiting dystrophin expression [25]. Prednisolone administration decreases muscle levels of miRNAs related to infl (miR-142-5p, miR-142-3p, miR-146a, miR-301a, miR-324-3p, miR-455-5p, miR-455-3p, miR-497, miR-652) up to normal values in the murine *mdx* model [40] and affects serum expression of selected miRNAs (miR-206, miR-181a, miR-4538, miR-4539, miR-606, miR-454) altered in young DMD patients [41]. Thus, another possible mechanism of action of corticosteroids may be related to serum miR-206 reduction, in which early overexpression negatively regulates target genes (RHGAP31, KHSRP, CORO1B, PTBP1, C7ORF58, DLG4, KLF4) worsening motor function in DMD [41]. Indeed, steroid treatment seems to further increase miR-1 and miR-133 levels in DMD, especially on a daily regimen compared to an intermittent one [42]. Moreover, miR-146 expression can be activated by NFkB and has a role in the immune system [43].

Fibrosis Another relevant feature of muscular dystrophies is collagen deposition. Studies on murine models identified miR-1 and miR-29 as being related to the TGF-β pathway, fibrosis and myogenic differentiation.

Myoblast differentiation and myotube formation are promoted by miR-29 acting on *Akt3*, a serine/threonine protein

Table 1 Role in skeletal muscle tissue and alteration of the most relevant miRNAs and lncRNAs in dystrophinopathies in muscle (M) and serum (S) samples of DMD or BMD patients or animal models

ncRNA	Role in skeletal muscle	Dystrophinopathies		References
miR-1	Promote muscle differentiation Role in FAP switch (S) Degeneration	Upregulated (S) Downregulated (M)	DMD*mdx*	[39, 128–132] [36]
miR-21	Promotes fibrosis	Upregulated (M)	*mdx*	[46, 47]
miR-23a	Inhibits differentiation	Downregulated (U)	DMD	[73, 142]
miR-29	Decreases fibrosis Promotes myoblast differentiation	Downregulated (M)	*mdx*	[43]
miR-31	Induced in ischemia damaged myofibers Expressed in quiescent satellite cells Dystrophin expression inhibitor Delay muscle differentiation	Upregulated (S) Upregulated (M)	DMD *mdx*	[23, 39, 65]
miR-34c	Regeneration Induced in ischemia damaged myofibers	Upregulated (M)	*mdx*	[36]
miR-133a	Promotes myoblasts proliferation Role in FAP switch (S)	Upregulated (S) Downregulated (M)	DMD BMD	[39, 128–132] [130]
miR-133b	Myoblast proliferation Role in FAP switch (S)	Upregulated (S)	DMD BMD	[39, 128–132] [130]
miR-146a	Promotes C2C12 myoblasts proliferation Dystrophin expression inhibitor	Upregulated (M)	*mdx* BMD	[23]
miR-146b	Promotes myogenic differentiation Dystrophin expression inhibitor	Upregulated (M)	*mdx* BMD	[23]
miR-195	Dystrophin expression enhancer	Upregulated (M)	BMD	[23]
miR-199a	Promotes fibrosis Proliferation and differentiation (WNT pathway)	Upregulated (M)	DMD	[48]
miR-206	Regeneration after muscle damage (expressed in proliferating myoblasts) Activation of satellite cells Promotes muscle differentiation Role in FAP switch (S)	Upregulated (S) Upregulated (M)	DMD BMD *mdx*	[39, 128–132] [130] [36]
miR-208b	Myostatin related	Upregulated (S)	DMD	[131]
miR-221	Progression from myoblasts to myocytes (expressed in terminally differentiated myoblasts)	Upregulated (M)	DMD BMD	[77]
miR-222	Inflammation	Upregulated (M)	GRMD	[55]
miR-223	Inflammation Dystrophin expression inhibitor	Upregulated (M)	*mdx* BMD	[23]
miR-320a	Dystrophin expression inhibitor	Upregulated (M)	BMD	[23]
miR-374a	Dystrophin expression inhibitor	Upregulated (M)	BMD	[23]
miR-382	Dystrophin expression inhibitor	Upregulated (M)	*mdx* BMD	[23]
miR-486	Muscle growth and apoptosis Promotes differentiation	Downregulated (M)	GRMD	[55]
miR-494	Regeneration	Upregulated (M)	*mdx*	[23]
miR-499	Mithocondrial function	Upregulated (S)	DMD	[131]
miR-758	Dystrophin expression enhancer	Upregulated (M)	BMD	[23]
SRA	Muscle differentiation	Downregulated (M)	*mdx*	[81, 98]
RAM	Activation of myogenesis	?	?	[90]
Dum	Myoblasts differentiation	?	?	[86]
YY1	Myogenic differentiation Promotes muscle regeneration	?	?	[89]
Lnc-31	Counteracts differentiation	Upregulated (M)	*mdx* DMD	[96]
Lnc-MD1	Muscle differentiation and regeneration	Upregulated (M) Downregulated (M)	*mdx* DMD	[95, 98]
Lnc-mg	Increase IGF2	?	?	[93]

kinase responsive to growth factor cell signaling [44], and NFkB/Yin Yang 1 (YY1) [45] pathways. Moreover, miR-29 targets transcripts of fibrotic genes (for example, *COL3A1*, *FBN1*, *YY1*, *COL1A1*) and is downregulated in the muscle of *mdx* mice, to some extent due to TGF-β-mediated negative regulation. The loss of miR-29 in myoblasts promotes their differentiation in myofibroblasts and increases fibrosis [46].

MiR-1, expressed in adult skeletal and cardiac muscle tissue, promotes muscle diff entiation targeting HDAC4, which in turn inhibits skeletal muscle gene expression [47]. HDAC2, increased in DMD, represses both miR-1 and miR-29 expression in *mdx* mice; indeed, enhancing nuclear sphingolipid sphingosine-1-phosphate (S1P) inhibits HDAC in the *mdx* mice muscles, increasing miR-1 and miR-29 and reducing fibrosis [48].

Another miRNA implicated in skeletal muscle fibrosis is miR-21, which has been found to be increased in a dystrophic mouse model and DMD fibroblasts. MiR-21 targets *PTEN* and *SPRY-1* genes. Its levels positively correlate with *COL1A1* and *COL6A1* transcript levels, and it is regulated by the extracellular proteolytic inhibitor PAI-1 and enhanced by TGF-β. Muscle fibroblasts of the dystrophic mouse model present an imbalance between PAI-1 and urokinase-type plasminogen activator (uPA), favoring the activation of TGF-β and miR-21 expression. In *mdx* mice, muscle fibrosis is anticipated by the loss of PAI-1 or the overexpression of miR-21, while it is reduced by miR-21 inhibition [49, 50]. Furthermore, the fibrotic response is promoted by exosomes containing miR-199a-5p produced by DMD muscle fi oblasts [51].

Recent studies have highlighted the role of fibro-adipogenic progenitors (FAPs) in the pathogenesis of DMD, supporting regeneration during the early stage of the disease and fibro-fatty replacement in the late phase. Experiments on the murine *mdx* model demonstrated that histone deacetylase inhibitors (HDACi) promote muscle cell regeneration and prevent connective substitution only if administered in earlier stages, facilitating the myogenic phenotype. Instead, in the late stage of the disease, the fibro-adipogenic phenotype of FAPs prevails. Selected myomiRs (miR-1, miR-133, miR-206), induced by HDACi, seem to be implicated, through BAF60a and BAF60b, in the switch between fi o-adipogenic and myogenic phenotype of FAPs [52, 53].

Nitric oxide pathway The absence of dystrophin also brings about delocalization and reduction of nitric oxide synthase (nNOS) levels, leading to increased muscle fatigability. NO is responsible for the S-nitrosylation and chromatin association of HDAC2, which results in increased activity in *mdx* mice. As previously stated, altered HDAC2 modifies the expression of fibrosis-related miRNAs (miR-1 and miR-29), while miR-206 is not aff since it is expressed in activated satellite cells [54]. Analysis of 617 BMD (Δ45-55) muscular biopsies revealed overexpression of miR-31, miR-708 and miR-34c interacting negatively with nNOS; in DMD myoblasts, inhibition of miR-708 and miR-34c increased nNOS expression, confirming their role in nNOS modulation [55]. Moreover, nitric oxide upregulates miR-27b, and decreasing peroxisome proliferator-activated receptor gamma expression, inhibits the diff entiation of FAPs into adipocytes [56].

Disruption of the DAPC Dystrophinopathies may also have a secondary defi of the other components of the dystrophin–glycoprotein complex. De Arcangelis et al. suggested the role of the overexpression of miR-222 in this phenomenon, since it binds β1-syntrophin 3′UTR in the *mdx* mouse skeletal muscle with consequent downregulation of this protein [57]. To support this hypothesis, Robriquet et al. demonstrated that miR-222 and miR-486 are upregulated in newly regenerated muscle fi of GRMD dogs [58].

Myogenesis Muscle fi originate from mesodermic myogenic committed progenitors, expressing Pax3 and Pax7 and turn into myoblasts that proliferate, diff entiate and fuse into mononucleated myocytes and then multinucleated myotubes. A subpopulation forms the skeletal muscle satellite cells (SCs), mononucleated cells localized between the sarcolemma and basal lamina of muscle fibers, that remain quiescent in the adult muscle, but are able to activate and proliferate in response to muscle injury [59]. Pax7 is fundamental for the maintenance and proliferation of SCs and is repressed with the progression of myogenesis [60]. Both embryonal and adult myogenesis depends on the expression of myogenic regulatory factors (MRFs—MyoD, Myf5, myogenin and MRF4) and the interaction between each other. The myogenic determination factor 1 (MyoD) and myogenic factor 5 (Myf5) are expressed in early stages and involved in commitment and proliferation of the myogenic directed cells, while myogenin and herculin (MRF4) induce the terminal differentiation of committed cells with the subsequent fusion of myocytes and formation of myotubes. However, even MyoD can be involved in the regulation of terminal differentiation, and MRF4 also has a role in the early commitment stage. Moreover, myocyte enhancer factor 2 (MEF2) proteins are able to positively regulate different MRFs. Other factors involved in the regulation of myogenesis are HGF, FGFs, IGF-1 splice variants, myostatin, BMP4, Wnt proteins, Six1, Six4 and TGF-β. [61–64]. The complex process of myogenesis and the role of MRFs and ncRNAs in this process is represented in greater detail in **Fig. 1**.

MiRNAs are involved in the regulation of myogenesis, reciprocally interacting with myogenic transcription factors such as Pax3/7, Myf5, MyoD, myogenin, MRF4, MEF2 and SRF [65, 66]. Muscle-specifi lncRNAs are mainly expressed during myoblast differentiation and regulate transcription factors implicated in myogenesis, including MyoD and Myf5, as well [67].

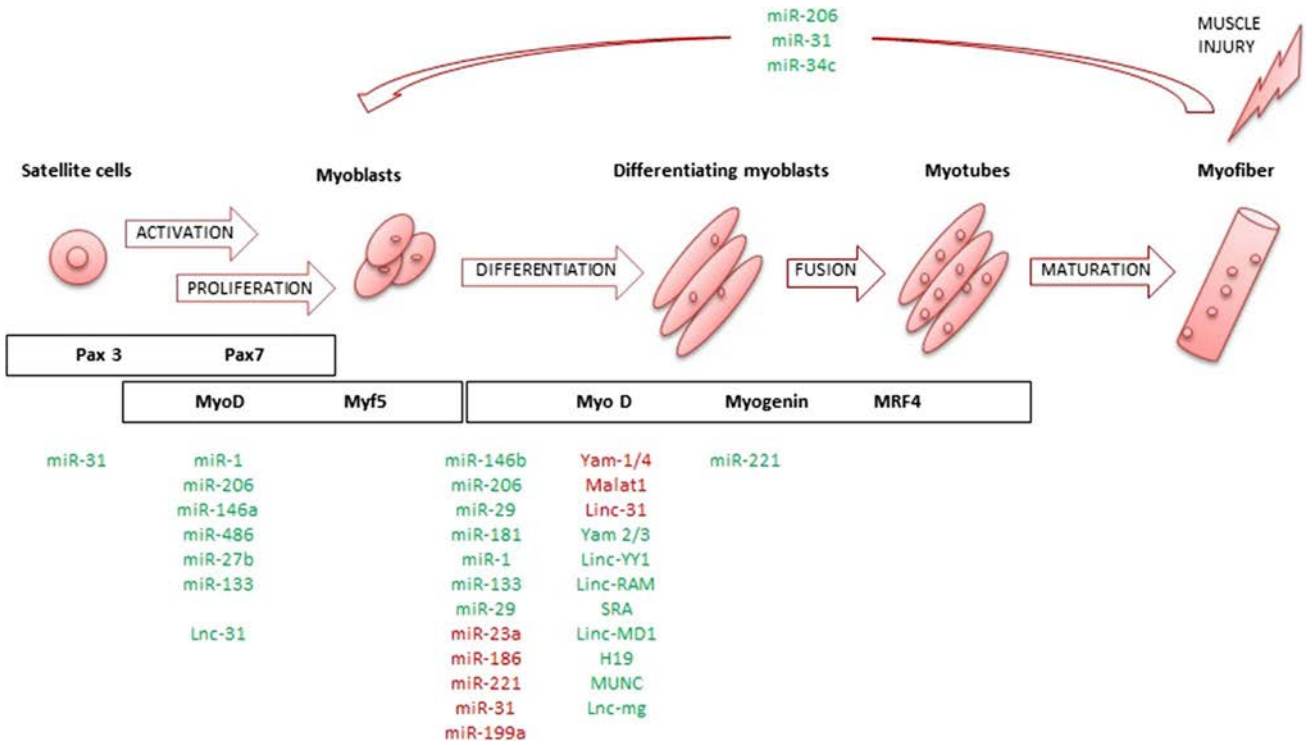

Fig. 1 Pax3 and Pax7 maintain satellite cells in a quiescence state by negatively regulating MyoD; miR-31 is involved in the maintenance of quiescence. Activation of muscle stem cells downregulates Pax3/7 so that Myf5 and MyoD are upregulated and involves several miRNAs. This leads to myoblast proliferation. After cell cycle exit, myogenesis implies differentiation of myoblasts, fusion to form myotubes and maturation into myofibers. Other ncRNAs participate in response to muscle injury. In the scheme have been shown the main ncRNAs facilitating (green) or inhibiting (red) the various phases of myogenesis

In satellite cells and regenerating myoblasts, miR-31 is highly expressed and seems to suppress the translation of transcripts for early myogenic factors targeting Myf5 and maintaining SCs in a quiescent state. DMD biopsies showed more elevated levels of miR-31 than healthy controls [68]. On the contrary, miR-1 and miR-206 facilitate the differentiation of SCs targeting Pax7 mRNA and thus blocking their proliferation [47]. Pax7 expression in SCs is also enhanced by a pathway involving tumor necrosis factor (TNF) receptor associated factor 6 (TRAF6) and c-JUN/activator protein 1 transcription factor. Lack of TRAF6 leads to overexpression of miR-1, miR-133 and miR-206 in cultured myogenic cells and subsequent too early diff entiation of SCs [69]. Also, miR-27b promotes SCs differentiation targeting Pax3 mRNA, and is induced at the beginning of the myogenic differentiation program [70].

Myoblast proliferation can be induced by miR-146a [71], miR-1, that targets HDAC4, and miR-133, which represses serum response factor (SRF) [47].

Other ncRNAs are involved in later stages of myogenesis. For instance, miR-146b [72] and miR-206 [73] promote myogenic differentiation targeting Smad Family Member 4 (Smad4) and Notch1 and p180 subunit of DNA polymerase alpha, respectively. Downregulation of miR-29, as reported in *mdx* muscle, increases Ying Yang-1 (YY1) in C2C12 myoblasts, diminishing the differentiation of myoblasts into myotubes [74]. Furthermore, miR-181 increases terminal differentiation targeting the repressor Hox-A11 [75].

On the contrary, miR-23a inhibits myoblasts differentiation, acting on Myh1, Myh2 and Myh4 [76]. Mir-186 targets myogenin in differentiation myoblasts and is repressed during myogenic differentiation [77]. Diminished myoblast differentiation can also be induced by transforming growth factor (TGF) β1 that represses miR-24, miR-29 and miR-206 [78]. Even miR-221 participates in the progression from myoblasts to myocytes and its expression causes a delay in withdrawal from the cell cycle and the inhibition of sarcomeric protein accumulation in differentiating myoblasts [79]. MiR-221 is upregulated in DMD and BMD [80], suggesting a delay in the diff entiation process. Likewise, miR-31 is implicated in terminal differentiation, interacting with both Myf5 and dystrophin. While miR-31 levels tend to decrease at later stages of differentiation in wild-type mouse satellite cells, they are persistently upregulated in muscle tissue cultures of *mdx* model [81] and DMD myoblasts [68], causing a delay in muscle differentiation. MiR-199a-3p targets IGF1/AKT/mTOR pathway partially blocking myoblasts differentiation in C2C12 myoblasts [82].

Also, lncRNAs act during myoblasts diff entiation, as previously stated [67]. eRNAs *CE* and *DRR* regulate MyoD and myogenin, modifying the access to chromatin and the recruitment of RNAP II [83]. *SRA* lncRNA and its protein isoform SRAP facilitate MyoD activity in opposite ways, with an increase in the SRA/SRAP ratio during myogenesis and consequent activation of MyoD [84]. MyoD, in turn, controls *H19*, a lncRNA highly expressed during embryonic development that represses MyoD-inhibiting protein IGF2. *H19* has a pro-myogenic role and promotes skeletal muscle differentiation and regeneration encoding miR-675-3p and miR-675-5p, which, respectively, repress a pathway mediated by bone morphogenetic protein (BMP) and the DNA replication initiation factor Cdc6 [85–87]. MyoD also targets *lnc-AK143003* [88] and *lcnRNA Dum* [89]. *Yam-1* (YY1-associated muscle 1) and Yam-4 inhibit muscle differentiation, while Yam-2 and Yam-3 promote differentiation. Notably, Yam-1 reduces myomiRs expression during proliferation and represses myogenesis by activating miR-715 [90]. Deletion of *Malat1* in *mdx* mice improves muscle regeneration since it delays the switch between proliferative and differentiative phases of myogenesis; upon differentiation, miR-181a targets *Malat1* for degradation, allowing MyoD activation [91]. *Linc-YY1* promotes both myogenic differentiation and muscle regeneration [92]. *Linc-RAM* (linc-RNA activator of myogenesis) is upregulated during myogenesis and enhances transcription myogenic genes, including miR-206, by directly binding MyoD [93]. *MUNC* (MyoD upstream noncoding) favors myoblast differentiation by increasing MyoD, myogenin and Myh3 mRNA expression [94]. In contrast, *Sirt1 AS lncRNA* inhibits differentiation but promotes myoblast proliferation interacting with miR-34a [95]. *Lnc-mg* acts as a miR-125b sponge, increasing insulin-like growth factor 2 (IGF2), and its levels rise during myogenic differentiation. Lack of *lnc-mg* (myogenesis-associated lncRNA) results in muscle atrophy and loss of endurance in knockout mice, while its overexpression leads to muscle hypertrophy [96]. Muscle anabolic regulator 1 (MAR1) acts as a sponge for miR-487b regulating the myogenic regulator Wnt5 and its overexpression can increase muscle mass [97].

The expression of certain lncRNAs is altered in muscular dystrophies. In DMD myoblasts, Cesana et al. detected diminished levels of *linc-MD1* that correlate with retardation of the muscle diff entiation program. In fact, *linc-MD1* binds miR-133 and miR-135, acting as a competing endogenous RNA for their mRNA targets, including the transcription factors MAML1 and MEF2C, which encode crucial myogenic factors. *linc-MD1* administration appears to partially rescue the abnormal kinetics of differentiation in vitro [98]. Ballarino et al. identifi *lnc-31* as a lncRNA counteracting differentiation in proliferating myoblasts. Indeed, its expression is higher in those cells and is downregulated during diff entiation. *Lnc-31* is also more abundant in *mdx* muscles and DMD myoblasts due to a more intense regenerative activity; delay in the differentiation program in DMD myoblasts relates with less pronounced downregulation of *lnc-31*. Notably, *lnc-31* originates from the precursor of miR-31 in an independent way [99].

Regeneration and response to myofi damage involve several miRNAs. For instance, miR-1, miR-133 and miR-206 accelerate muscle regeneration, if injected in mouse models of muscle injury, inducing MyoD, myogenin and Pax7 [100]. Butchart et al. demonstrated a dysregulation of certain ncRNAs in *mdx* mice compared to C57 mice. During the pre-necrotic phase, at 2 weeks, Meg3 and lncRNA SRA levels were lower, while miR-206 muscle levels were higher in *mdx* mice. On the other hand, during the necrosis and regeneration stage, spanning from 4 to 6 weeks, linc-MD1 levels markedly increased instead of decreasing, while miR-1, miR-133a and Neat1_v2 levels were lower than C57 mice [101].

Other miRNAs involved in myogenesis include miR-26a, regulating SMAD transcription factors, miR-221, miR-222, miR-322, miR-424 and miR-503, controlling cell-cycle regulators [65].

Cell signaling Even more signaling pathways implicate miRNA control. Ngheim et al. suggested that the upregulation of myostatin-related miRNAs (miR-539 and miR-208b) might play a role in hypertrophy and functional sparing of the cranial sartorius in GRMD [102].

MiR-486 is another muscular miRNA that facilitates myogenic differentiation, notably reduced in *mdx* mice and DMD patient muscles, whose overexpression improved performance and integrity of the sarcolemma, increased fi size and reduced nuclear centralization in *mdx* mice. One of the miR-486 targets in skeletal muscle is DOCK3, which is induced in dystrophic muscles and modulates the PTEN/AKT (phosphatase and tensin homolog deleted on chromosome 10/ankyrin1) signaling pathway, consequently regulating muscle growth and apoptosis [103, 104].

Myogenic regulatory factors of the WNT signaling pathway, involving cell proliferation and differentiation (FZD4, JAG1, WNT2), are targeted by miR-199a-5p. It is regulated in a serum response factor (SRF)-dependent manner and is overexpressed in animal models and human dystrophic muscle, causing myofi disruption and sarcolemmal detachment [105].

Another signaling pathway coupling mitochondrial function and muscle fi type is mediated by miR-499. This miRNA inhibits Fnip1 reactivating a pathway mediated by AMP-activated protein kinase (AMPK) and PGC-1a; restoration of miR-499 in the *mdx* mouse model lessens DMD severity [106].

Other miRNAs involved in various muscle regeneration and secondary response pathways have been found to be dysregulated in DMD muscle samples by Eisenberg et al. [80].

Other pathways Another subset of lncRNAs (Bvht and Fendrr) is also implicated in cardiomyocyte differentiation [107]. Cardiac muscle in dystrophinopathies presented a diff ent pattern of miRNA expression. For example, miR-448-3p downregulation is related to cellular oxidative stress [108] and miR-340-5p expression to eccentric cardiac hypertrophy [109]. The miR-143/β-dystrobrevin/synapsin I pathway is likely involved in neuronal diff entiation and, therefore, in brain involvement in dystrophinopathies [110]. Furthermore, lncRNA KUCG1, originating from chromosome X, could be related to mental retardation seen in some DMD patients [111].

Micropeptides Novel micropeptides, molecules smaller than 100 aminoacids, involved in muscle functions, have been recently discovered originating from muscle-specific lncRNAs [112, 113], posing the basis for further investigations of their role in dystrophinopathies. Here, a quick overview among micropeptides so far known as involved in muscle functions. Myoregulin (MLN), one of the most abundant transmembrane microproteins in adult skeletal muscle, controls the activity of SERCA, a membrane pump controlling calcium-mediated muscle relaxation [114]. Dwarf Open Reading Frame (DWORF) is highly expressed in the murine heart and enhances SERCA pump activity interacting with the inhibitory peptides phospholamban, sarcolipin, and MLN [115]. Furthermore, it has been demonstrated that micropeptide in mitochondria (MPM) is downregulated in DMD patients. This micropeptide is localized in mitochondria and upregulated during C2C12 myoblasts differentiation and regeneration after cardiotoxin damage [116]. Mitoregulin (Mtln) is another molecule, encoded by LINC00116, localized in the mitochondrial membrane, that binds cardiolipin, participates in protein assembly processes and enhances respiratory efficiency [117]. Instead, LINC00961 encodes for a small regulatory polypeptide of aminoacid response (SPAR), a lysosomal protein involved in mTORC1 signaling pathway and, therefore, protein synthesis and cell growth [118]. Myomaker and Myomixer are other two molecules that regulate myoblasts or myocytes fusion [119]. At last, lncRNA-Six1 interact with muscle-related genes (*MYOG, MYHC, MYOD, IGF1R, INSR*) and is involved in muscle growth, since its overexpression induces Six1 gene expression, through repression of miR-1611, enhancing cell proliferation and division [120, 121].

Serum biomarkers

An abnormal serum expression of diff ent miRNAs has been associated with various oncological, neurodegenerative, cardiovascular, metabolic and hereditary diseases, suggesting a potential role as a minimally invasive biomarker [122]. MiRNAs selectively involved in muscular pathways and related to muscular dystrophies have been named *dystromirs* or *myomiRs*. They include miR-1, miR-133a, miR-133b, miR-31 and miR-206 [123].

MiR-1 and miR-133a are expressed from the same transcript within the skeletal and cardiac muscle, but they have diff ent functions [80]. MiR-1 promotes myogenesis and terminal differentiation, acting on HDAC4 and connexin-43, while miR-133 enhances myoblast proliferation [47]. Mi-206 and miR-133b are also codified by the same ncRNA. MiR-206 is specifi to skeletal muscle, particularly oxidative fi and is expressed in proliferating myoblasts under negative regulation of TGF-β and myostatin and positive of MyoD and myogenin. MiR-206 promotes regeneration after muscle damage through differentiation and fusion of myotube progenitors, partially through HDAC4 inhibition; in fact, newly formed muscle fibers from satellite cells during regeneration in CTX-injured and dystrophic *mdx* muscles presented high expression of miR-206; the lack of this in CXMDJ muscles might be related to the potential of muscle regeneration. Moreover, miR-206 has a role in muscle hypertrophy and atrophy and suppresses utrophin, while its overexpression causes upregulation of utrophin levels in dystrophic conditions and decreases proinflammatory cytokines and macrophagic infi ation in *mdx* mouse muscle [124–128].

At fi t, it was assumed that myomiRs were passively released from damaged muscle fibers into the bloodstream after necrosis or sarcolemmal dysfunction. However, muscle levels of miRNAs do not always correspond to a proportional change of miRNA muscle levels in serum. Therefore, more complex mechanisms have been postulated, such as abnormal secretion due to dystrophin deficiency or selective release during muscle differentiation and regeneration [129]. Moreover, extracellular myomiRs seem to be predominantly non-vescicular [130].

A substantial increase in miR-1, miR-133a, miR-133b, and miR-206 has been demonstrated in the serum of patients with Duchenne muscular dystrophy [42, 131–135]. Discordant results involve serum levels of miR-31 in DMD since it has been found to be slightly increased by Zaharieva et al. [42] and decreased by Vigner [131].

A straightforward relationship with clinical features and disease progression has not yet been found. A few studies indicated an inverse correlation between serum levels of miR-1, miR-133 and miR-206 in ambulant DMD patients and disease severity evaluated through the Medical Research Council (MRC) scale, temporized tests [132] or North Star Ambulatory Assessment (NSAA) scale [133]. Instead, Zaharieva et al. found lower dystromir levels in the serum of patients having lost ambulation compared to ambulant subjects and subjects requiring scoliosis surgery or having

low forced vital capacity (FVC) values; no significant correlation with the NSAA score was reported [42]. These discrepancies may be partially explained by the different study populations analyzed regarding clinical severity and age. In fact, Cacchiarelli et al. evaluated patients from 3 to 6 years of age [133], Vignier et al. over 3 year-old [131], Zaharieva et al. ambulant DMD subjects between 4 and 13 years old [42]. Even the relationship with age differs in the various studies, either as being absent [132] or directly [133, 134] or inversely proportional [42] to serum levels. In one report, a considerable decrease in the amount of miR-133a, miR-133b, miR-31 and miR-1 was detected after the age of 11 years [42].

Furthermore, Zaharieva et al. found higher levels of miR-1, miR-31 and miR-133b in patients on a daily steroid regimen compared to intermittent regimen or absence of steroid treatment, suggesting a correlation between the anabolic effect of corticosteroids, increased muscle mass in subjects treated with higher steroids doses and circulating miRNAs levels [42].

Likewise, patients affected by BMD still present a mild increase in serum levels of miR-206, miR-1 and miR-133 in comparison to healthy controls, even if they are lower than in DMD [133]. Similar fi ngs have been described by Matsuzaka et al. [135] and Li et al. [134], but without statistical significance, in a group of patients between 5 and 31 years and 1 and 14 years, respectively. Patients studied by Li et al. were not on steroids. However, Zaharieva did not observe any difference in dystromir serum levels compared to controls, except for a slight increase in miR-206 [42].

Serum miR-206 is raised even in female carriers of dystrophinopathies [136]. Mouse *mdx* [131, 137] and canine X-linked muscular dystrophy ($CXMD_J$) models [138] showed overexpression of serum miR-1, miR-133a and miR-206.

Muscle levels of myomiRs can actually be unchanged or mildly increased or even decreased. For instance, Zaharieva showed that miR-1 is downregulated, while miR-206, miR-133a and miR-133b do not show signifi changes, confirming that serum levels are the result of more complex molecular mechanisms than passive release [42]. Moreover, miR-1 and miR-133 are abundant in mature muscle fibers but reduced in human and murine dystrophic myoblasts [54], while miR-206 is upregulated in skeletal muscles of 8 weeks old *mdx* mouse [137]. Deng et al. obtained muscle samples from a 1-month-old *mdx* mice highlighting overexpression of miR-1, miR-133a and miR-206 [139].

Thus, some authors suggested using miR-206 as a marker of muscle regeneration, considering that it is expressed in satellite cells and proliferating myoblasts and it decreases with age in muscle controls, and miR-1 and miR-133 are markers of residual muscle mass and decrease with age [42, 133, 134]. Extracellular myomiR levels can also be influenced by other factors, such as total muscle mass, exercise, muscle regeneration, immobilization and age [129, 140]. An increase in serum levels of miR-1, miR-133a and miR-206 was detected after muscle damage induced by CTX in mice [135]. Moreover, Gomes et al. demonstrated an increase in dystromir serum levels as a result of exercise in healthy subjects [141].

Other serum miRNAs altered in dystrophinopathies include miR-499, miR-208a, miR-208b [134], miR-30c, miR-181a [142], miR-378 [131], miR-95, miR-539 [143], miR-22 and miR-193b [144].

MiRNAs have been quantified even in different biological fl ids. The urine of 54 Duchenne muscular dystrophy patients displayed downregulation of miR-29c-3p (ambulant subjects), miR-23b-3p and miR-21-5p (nonambulant subjects) compared to healthy controls [145].

Structural cardiac alteration indicative of myocardial fibrosis correlates with serum increases of miR-222, miR-26a and miR-378a-5p [146]. Other miRNAs related to cardiomyopathy are miR-208a, miR-208b and miR-499, expressed mainly in cardiac muscle and slow skeletal muscle [134, 143].

Tool for therapy efficacy evaluation

Currently, several molecular approaches have been developed to treat dystrophinopathies. Most of the current clinical trials employ functional tests, such as the Six Minute Walking Distance or Motor Function Measure Scale, as an outcome measure. Unfortunately, a signifi change in functional clinical outcomes requires long treatment times.

Several potential biomarkers have been suggested, concerning myonecrosis, regeneration, inflammation, fibrosis, oxidative stress, both using histological analysis (i.e. albumin levels related to sarcolemmal leakiness, embryonic or neonatal myosins, central myonuclei, infl ory infi - trates) and serum or urine levels of different metabolites (i.e. CK, aldolase, fi C, myosin light chain, serum TNF) [147]. Changes in miRNA levels in muscle specimens or serum could be a promising and non-invasive tool to evaluate the efficacy of novel treatments on dystrophin expression, muscle damage and inflammation.

So far, the majority of these analyses was performed investigating exon skipping effects. Studies on the *mdx* murine model demonstrated an almost complete normalization of increased miR-1 and miR-206 serum levels (previously 20–40-fold compared to wild type), proportional to dystrophin restoration, after 1 month from the administration of exon 23 skipping treatment through adeno-associated virus (AAV) vectors [133]. Similar, but not statistically significant, myomir modifications were detected in the serum of DMD patients treated for 12 weeks with a novel morpholino

antisense oligomer inducing exon 51 skipping (Eteplirsen) [42]. Moreover, correct nNOS localization after exon 45 skipping in DMD myoblasts normalizes miR-1 and miR-29c expression [148]. In the *mdx* mouse, an increase in dystrophin levels after exon 23 skipping through endovenous morpholino administration corresponds to reduced levels of selected miRNAs in the anterior tibial muscle (miR-146a, miR-374a, miR-223, miR-320a, miR-382) but not in diaphragm or gastrocnemius muscles [26]. Also, Cacchiarelli et al. demonstrated how exon 23 skipping using AAV vectors in *mdx* mice resulted in normalization of downregulated miRNA (miR-1, miR-133, miR-29c, miR-30c), increase in miR-206 and reduction in miR-223 in muscle samples [54]. Roberts et al. showed normalization of serum miR-1, miR-133a and miR-206 after morpholino-mediated dystrophin restoration in *mdx* mice, proportional to the degree of protein rescue [137, 144]. Further studies found restoration towards wild-type levels of miR-21, miR-29c and miR-146b in *mdx* mouse muscles treated with exon skipping, but not of the upregulated miR-31, miR-34c and miR-206 [149].

A reduction in miR-31 levels, linked to dystrophin restoration, was observed after CRISPR/Cas9 treatment of myotubes derived from induced pluripotent stem cells (iPSCs) of DMD subjects [150].

Potential therapeutic targets

As shown by the following preliminary experiments, modulation of miRNA levels may be used as support for other innovative treatments.

One of the most consistently reported miRNAs involved in dystrophin expression is miR-31, which represses *DMD* expression targeting the 3′UTR region. MiR-31 showed a 50-fold enrichment compared with the control in *mdx* mouse muscle. Cacchiarelli et al. attempted exon 51 skipping in human DMD myoblasts using antisense sequences administered through lentivirus: concomitant inhibition of miR-31, though a specifi sponge construct, improved dystrophin restoration leading to dystrophin levels greater than those achieved by exon skipping alone [68]. Analogous studies were performed by Hildyard et al. injecting both exon skipping and miR31-modulating oligonucleotides in the tibialis anterior muscle of the *mdx* mouse. While their results were promising in cell culture models, several problems arose in vivo, since the two oligonucleotides competed with each other for myofi entry and intravenous delivery of skipping morpholino resulted in fatalities [151].

Supplementation of miR-29c, which is involved in muscle fibrosis, through the AAV vector in the gastrocnemius of the *mdx* mouse, leads to muscular connective tissue decrease and weakness improvement. Almost complete normalization of muscle strength has been obtained with the combined administration of miR-29c and microdystrophin [152]. Wang et al. detected an increase in the number of regenerating fibers after injection of oligonucleotides mimicking miR-29c in the tibialis anterior muscles of *mdx* mice [46].

Local injection of a mixture of miRNAs (miR-1, miR-133, miR-206) in a rat skeletal muscle injury model enhances muscle regeneration [100]. In contrast, Bulaklak et al. showed improved motor function in *mdx* mice after AAV-mediated administration of a miR-206 decoy target [153].

Utrophin is a protein analogue to dystrophin, able to partially compensate in its absence. This compensatory mechanism differs in mice and humans, due to differential regulation of *Utrn* isoforms, and provide a major protective effect in *mdx* mouse model, responsible for the milder phenotype compared to DMD patients [154]. Utrophin expression can be downregulated by miR-206, miR-150, miR-196b, miR-296-5p, miR-133b and let-7c; blocking those miRNAs resulted in increased levels of utrophin in C2C12 cells [155]. Blocking the interaction between let-7c and utrophin 3′UTR through oligonucleotides brings about utrophin upregulation and improvement of the dystrophic phenotype in the *mdx* mouse model [156].

Other noncoding RNAs may be employed for therapeutic purposes: U1 snRNA-derived antisense molecules induce exon 45 skipping in Δ44 human DMD myoblasts, relocalizing nNOS and recovering the normal expression of myogenic markers and selected miRNAs [148].

Last, Aminzadeh et al. developed an approach based on cardiosphere-derived cells (CDCs) injected into the hearts of *mdx* mice to improve survival and ambulatory capacity due to the secretion of exosomes able to increase dystrophin expression through miR-148a [157].

Perspectives and conclusions

Serum creatine kinase (CK) levels, an enzyme released by damaged muscle fi are elevated in the early stages of these muscular dystrophies and gradually decrease later on. Due to their fl they do not represent a precise blood biomarker for evaluating disease progression and clinical worsening or improvement. MiRNAs seem to be more stable than CKs and are correlated to clinical severity in some cases, suggesting a potential role as serum biomarker even in clinical trials, especially miR-1, miR-133 and miR-206. Further studies are needed to refi and standardize miRNA quantification on serum [158], and we should keep in mind that serum levels of myomiRs may increase both for muscle mass reduction and function improvement, as well as decrease both for physical activity and muscle mass increase [129].

Moreover, the analysis of miRNA expression may improve our knowledge of the pathogenesis of dystrophinopathies and the pathways upon which the drugs act. Different miRNA expression could also explain the different phenotype and dystrophin levels highlighted in BMD patients carrying the same deletion, since some of them are capable of dystrophin expression inhibition, as miR-31 in DMD patients.

MiRNAs can also be useful for monitoring the effi through serum or muscle changes, as reported mainly in studies involving exon skipping approaches. Furthermore, their administration may enhance the effects of known therapies, as shown with the administration of miR-31 modulating agents in addition to exon skipping, and improve pathological aspects of the disease, such as inflammation or fibrosis, as reported by Heller et al. with the supplementation of miR-29 and microdystrophin in *mdx* mice [152].

In conclusion, miRNAs have been demonstrated to be an interesting research field in dystrophinopathies, regarding pathogenesis, progression of the disease and treatment. While little is yet known, the increasing discoveries related to roles of lncRNAs in muscle functions and their interaction with miRNAs, are likely to provide further insights for dystrophinopathies.

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