Title

Biomarker Potential of Extracellular MicroRNAs in Duchenne Muscular Dystrophy

Authors

Anna M. L. Coenen-Stass[1,2], Matthew J. A. Wood[1*], and Thomas C. Roberts[1,3*]

Affiliations

[1]Department of Physiology, Anatomy and Genetics, University of Oxford, South Parks Road, Oxford, OX1 3QX, UK
[2]Institute of Neurology, University College London, London, UK
[3]Sanford Burnham Prebys Medical Discovery Institute, Development, Aging and Regeneration Program, La Jolla, CA, 92037, USA
*To whom correspondence should be addressed

Corresponding Authors

Professor Matthew J.A. Wood
Department of Physiology, Anatomy and Genetics
University of Oxford
South Parks Road
Oxford
OX1 3QX
United Kingdom
Telephone   +44 (0)1865 272419
Fax   +44 (0)1865 272420
Email   matthew.wood@dpag.ox.ac.uk

Dr Thomas C. Roberts
Sanford Burnham Prebys Medical Discovery Institute
Development, Aging and Regeneration Program
10901 N Torrey Pines Rd
La Jolla
CA 92037
USA
Telephone   +1 858 646 3100 x3225
Fax   +1 858 795 5298
Email   thomas.roberts@dpag.ox.ac.uk

Abstract

MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression within cells, but might also constitute promising extracellular biomarkers for a variety of pathologies, including the progressive muscle-wasting disorder Duchenne Muscular Dystrophy (DMD). A set of muscle-enriched miRNAs, the myomiRs (miR-1, miR-133, and miR-206), are highly elevated in the serum of DMD patients and dystrophin-deficient animal models. Furthermore, circulating myomiRs might be used as pharmacodynamic biomarkers, given that their levels can be restored towards wild-type levels following exon skipping therapy in dystrophic mice. The relationship between muscle pathology and extracellular myomiR release is complex, and incompletely understood. Here we discuss current progress towards the clinical utility of extracellular miRNAs as putative DMD biomarkers, and their possible contribution to muscle physiology.

Keywords

Biomarkers, microRNA, extracellular vesicles, extracellular RNA, exosomes, Duchenne Muscular Dystrophy

Biomarkers of Duchenne Muscular Dystrophy: An Unmet Clinical Need

Duchenne Muscular Dystrophy (DMD) is an X-linked, monogenic, muscle-wasting disorder and the most prevalent inherited myopathy affecting children [1,2] (**Box 1**). Although currently incurable, DMD is a prime candidate for regenerative medicine and gene therapy approaches, the most advanced of which is **antisense oligonucleotide-mediated exon skipping** (see glossary). This strategy aims to modulate the splicing of the *DMD* pre-mRNA transcript in order to restore the dystrophin translation reading frame, and in so doing, convert the DMD phenotype to the less severe condition, **Becker Muscular Dystrophy** (BMD) (**Box 2**). Indeed, the drug **eteplirsen** (designed to skip human *DMD* exon 51) was granted accelerated approval by the USA FDA in September 2016, pending further demonstration of efficacy [3] - a decision that has proven controversial [4–6]. Conversely, a competing compound **drisapersen**, was declined FDA approval in January 2016 after failing to meet its primary endpoint in a phase III clinical trial (NCT01254019) - effectively ending its further development [7,8]. In both cases, issues surrounding drug efficacy, lack of **surrogate biomarkers**, and the resulting difficulties in determining patient benefit were crucial factors in the decisions made by the regulators. These current events highlight the fact that the development of biomarkers for monitoring therapeutic efficacy has lagged behind that of experimental dystrophin restoration therapies and as such, there is great interest in the identification of minimally-invasive DMD biomarkers to address this unmet clinical need [9,10].

MicroRNAs (miRNAs) are one such class of potential biomarkers. These small RNA molecules have been implicated in normal muscle function, and dystrophic pathology (**Box 3**). In 2008, it

was demonstrated that **extracellular microRNAs** (ex-miRNAs) are present in **biofluids** such as human serum and plasma, and that extracellular miR-141 levels could be utilized as a biomarker to predict the presence of prostate cancer [11]. Numerous other studies have subsequently investigated circulating miRNAs in a wide variety of disease contexts [12]. Here, we review recent progress towards the potential clinical utility of ex-miRNA biomarkers for monitoring DMD pathology, assessing the pharmacodynamic response to novel therapies such as exon skipping, and the mechanisms which lead to ex-miRNA release. Lastly, we discuss the hypothesis that these molecules function to transfer gene regulatory information between cells in the muscle niche.

Current DMD Biomarker Strategies

Outcome measures in DMD clinical trials typically consist of (i) a muscle biopsy in which **dystrophin** expression can be measured by immunofluorescence and Western blot, and (ii) functional assessments, such as the distance that a patient can walk in 6 minutes (i.e. the **6 Minute Walk Test**, 6MWT) [13–16]. The highly invasive and painful nature of obtaining a muscle biopsy means that repeated measurements in DMD boys is ethically problematic. Furthermore, a biopsy samples only a small portion of a single muscle, which may not constitute a representative sample of that specific muscle, or the musculature overall. Assessment of dystrophin fiber positivity is somewhat subjective, and reproducibility is affected by the choice of antibody used, methodological differences between labs, conflicting definitions of fiber positivity, and inter-investigator variation [8,17]. Measurement of total dystrophin expression by Western blot is challenging as this protein is normally expressed at low levels, and this technique

4

can only be considered quantitative if patient samples are compared to a standard curve (e.g. dilutions of wild-type protein lysate).

Similarly, the use of the 6MWT to assess muscle function is also subject to several limitations. Specifically, the test is only applicable to ambulant patients by definition, and factors such as patient fatigue, motivation, coaching, non-compliance with the test protocol, and concerns regarding injury due to falling are all potential confounding factors. Indeed, in an eteplirsen clinical trial in 12 patients, two patients lost ambulation within 6 months of starting the trial and so were excluded as non-evaluable from the 6MWT analysis (these patients were identical twins, the oldest participants in the trial, and had the lowest baseline 6MWT scores) [16]. Trial data were therefore analyzed using a **modified-Intention-To-Treat** (mITT) approach, which is itself a controversial *post hoc* modification to the trial design which may bias the analysis towards detecting a positive result [6]. Nevertheless, all remaining trial participants treated with eteplirsen for 3 years maintained ambulation at least until the age of 13, indicating that these patients performed better than expected based on disease natural history [18]. Notably, while the 6MWT may not be sensitive enough to detect subtle changes over a short time-frame, it is informative when considering disease progression over multiple years.

Biofluid analysis is widely used in clinical pathology to diagnose and monitor disease as samples are readily accessible, and simple to collect. The most commonly used clinical biochemistry factor used to assess DMD patients is the muscle-specific isoform of Creatine Kinase (CK).

Elevated CK is observed in the serum of DMD patients throughout life, although levels gradually decline with age, likely as a consequence of a progressive reduction in muscle mass [19] and/or inactivity due to loss of ambulance [20]. Importantly, serum CK is a non-specific marker of muscle pathology which is elevated in a plethora of other neuromuscular disorders, and CK levels do not necessarily correlate with functional read-outs of muscle, such as Magnetic Resonance Imaging (MRI) [21].

The limitations of current endpoint measures have motivated the search for alternative DMD biomarkers. An ideal DMD biomarker, or panel of biomarkers, should fulfill the following criteria; (i) be easily and repeatedly measureable while being non-invasive or minimally-invasive for the patient, (ii) be able to distinguish between affected and unaffected individuals, (iii) act as a pharmacodynamic marker to monitor the efficacy of therapy, and (iv) predict clinical benefit [22].

Rationale for Considering ex-miRNAs as Putative DMD Biomarkers

ex-miRNAs constitute a rich pool of potential minimally-invasive biomarkers on account of their presence in a wide range of human biofluids (including serum and plasma). From a biochemical perspective, ex-miRNAs are ideal biomarkers as they are abundant, stable in stored biofluids samples [23], they are easily measured using low cost techniques (for detailed protocols for ex-miRNA quantification see references [24,25]), and ex-miRNA candidate discovery is facile (i.e. using hybridization, PCR amplification or deep sequencing methodologies) relative to equivalent efforts to identify novel protein biomarkers [26,27]. From a biological perspective, the use of ex-

miRNAs as biomarkers is advantageous on account of their tissue-specific expression patterns, and their regulatory roles in physiological/pathophysiological processes. For instance, changes in ex-miRNA concentrations might reflect alterations in distinct biological states, or damage to a specific organ or tissue. Indeed, miR-133a was identified as a sensitive biomarker of muscle damage as it was highly elevated (500-800 fold) in the plasma of Sprague Dawley rats exposed to the muscle toxicants 2,3,5,6-tetramethyl-p-phenylenediamine (TMPD) or Statin A [28]. In contrast, the levels of the brain and liver-specific miRNAs (which have no known association with DMD) miR-124 and miR-122 respectively, were unaffected [28].

Moreover, multiple groups have since reported elevated serum/plasma **muscle-enriched microRNAs** (**myomiR**) levels in DMD patients [29–36] (summarized in **Table S1**). The majority of these studies have focused on the classical myomiRs; miR-1, miR-133 and miR-206. Here we use the term myomiRs to refer specifically to these three miRNAs (unless otherwise stated). These myomiRs are all highly elevated in DMD patient sera and exhibit close to 100% specificity and sensitivity in distinguishing between DMD and healthy individuals [29–34]. Serum miR-206 was also reported to be significantly elevated in female DMD carriers relative to unaffected individuals, indicating that this miRNA may have utility for carrier detection [37]. Furthermore, sera from patients with the less severe dystrophinopathy, BMD, has been reported to contain serum myomiRs at intermediate levels between those of DMD patients and healthy controls [29,30,32], suggesting that ex-myomiR levels might potentially reflect disease severity and progression. Elevated circulating myomiR levels have also been reported in a variety of dystrophin-deficient animal models including the *mdx* **mouse** [38–41], *mdx*[4cv] **mouse** [31],

dystrophin/utrophin double knockout *dko* **mouse** [42], and dystrophic dog models (**CXMD**$_J$ [38] and **GRMD** [35]), relative to wild-type controls. Global serum miRNA profiling in dystrophic animal models, and studies focused on smaller subsets of miRNAs in human patient serum, have identified additional putative biomarkers associated with DMD, including: miR-22, miR-30, miR-95, miR-181, miR-193b, miR-208a, miR-208b, miR-378 and miR-499 [31,35,36,40]. However, extensive further testing is needed to validate these candidates as *bona fide* DMD biomarkers.

Attempts to relate ex-myomiR levels to other clinical parameters in DMD patients have so far produced some conflicting findings (**Table S1**). Several studies have reported a negative correlation between ex-myomiR levels and DMD patient age [29,30], similar to the negative correlation observed with serum CK [19]. Consistent with these human studies, aged *mdx* mice (88 week old) also exhibited reduced ex-myomiR levels relative to adult mice (14 week old), which corresponded to loss of muscle mass [41]. In contrast, ex-myomiR levels have been reported to increase with age in younger DMD patients (age 2-6) [33]; this might be explained by the fact that, over this age range, DMD patients undergo a period of normal childhood growth (and increased 6 minute walk distance) which may compensate for myofiber degeneration [43]. Accordingly, serum myomiR levels were significantly higher in ambulant (mean age 8.2 years) versus non-ambulant DMD patients (mean age 14 years), likely as a consequence of pathological progression and/or higher levels of physical activity [30]. Indeed, exercise in healthy individuals can also lead to an increase in circulating myomiRs [44], and serum myomiR levels in the *mdx* mouse are elevated following treadmill exercise [38,41]. Notably, the magnitude of ex-myomiR

elevation following acute exercise was much lower than that observed for serum CK [38,41], suggesting that the measurement of ex-myomiR levels are less sensitive to exercise-associated variability than serum CK [38,41].

Conversely, serum myomiR levels have been reported to be inversely correlated with **North Star Ambulatory Assessment** (NSAA) scores in a small cohort of 10 ambulant DMD patients (3-6 years old), indicating that, in this age group, lower ex-myomiR levels may be indicative of better functional performance [29]. However, a similar correlation was not reported in a larger patient cohort (n=26) with a broader range of ages (4-13 years) [30]. Consequently, further studies with greater numbers of patients are needed to fully elucidate the relationships between serum myomiR levels and metrics of muscle function (such as MRI and 6MWT) or age.

Collectively, these studies suggest that ex-myomiR levels are influenced by a number of factors which contribute to the complex pathology occurring in dystrophic muscle, such as changes in muscle mass, physical activity, and muscle growth/regeneration. As a result, in older patients with advanced pathology, serum myomiR levels alone may lack the capacity to monitor disease progression (or response to therapy), as a decrease in their levels could be indicative of either an improvement in muscle function or a reduction in muscle mass [30,41]. Conversely, higher serum myomiR levels might suggest an increase in pathology and muscle degeneration, but may equally be a consequence of muscle growth/regeneration and/or greater physical activity, as observed in younger patients [30,41]. An important goal for future studies will be to determine the extent to which these various factors contribute to ex-myomiR release.

9

ex-miRNAs as Putative Pharmacodynamic DMD Biomarkers in Preclinical Models and Clinical Trials

ex-myomiRs have been investigated as pharmacodynamic biomarkers in various pre-clinical models of DMD. For instance, therapeutic rescue of dystrophin protein expression using the U1 or U7 snRNA-based exon skipping systems restored serum miR-1 and miR-206 towards wild-type levels in *mdx* mice [29], and in the severely affected *dko* mouse [42]. Similarly, a non-viral exon skipping approach using a single systemic administration of peptide-conjugated antisense oligonucleotides (**Phosphorodiamidate Morpholino Oligmer** chemistry) also restored serum myomiR levels in the *mdx* mouse [39]. In addition, comparison of two peptide-oligonucleotide conjugates of different potencies revealed that higher levels of dystrophin re-expression in *mdx* muscle were associated with a greater degree of serum ex-myomiR restoration [40]. Although these observations suggest a dose-dependent relationship between ex-myomiR restoration and therapeutic efficacy, a direct correlation between muscle dystrophin expression and serum myomiR levels seems unlikely, considering that near complete restoration of circulating myomiR levels was observed in mice that exhibited incomplete recovery of dystrophin protein expression (~35% of wild-type levels) [40], although this level of dystrophin recovery is likely to be sufficient for therapeutic benefit [45]). As a result, we propose that ex-myomiR abundance may be a function of specific pathological features (e.g. **sarcolemmal stability**) which may be indirectly related to dystrophin expression, but not of dystrophin protein expression levels *per se*.

Building on these pre-clinical studies, ex-myomiR levels were further investigated in serum samples collected from DMD patients as part of two eteplirsen clinical trials; (i) a local delivery trial (intramuscular injection 0.09-0.9 mg, *n*=7 patients total) [13], and (ii) a systemic delivery trial (weekly intravenous injections for 12 weeks, 2-20 mg/kg, *n*=12 patients analyzed) [14]. In these patients, a trend towards serum myomiR restoration was observed following treatment, although this did not reach statistical significance [30]. It is likely that the amount of restored dystrophin was insufficient (and sample sizes too small) to achieve a statistically significant ex-myomiR restoration effect [13,14]. In further support of this notion, a separate eteplirsen trial in which patient biopsies were assessed by Western blot using a more rigorous methodology after 180 weeks of treatment revealed dystrophin expression to be only 1% of healthy controls [46] (no serum miRNA data are currently available for these patients). Achieving levels of dystrophin protein restoration in DMD patient muscle that are comparable to those possible in dystrophic animal models may be challenging using the current generation antisense oligonucleotide chemistries. Consequently, further trials with more potent exon skipping compounds and greater statistical power are required to validate ex-myomiRs as pharmacodynamic for DMD. Importantly, considering that circulating myomiR levels may change substantially as a consequence of aging and disease progression independent of any pharmacodynamic effects, their levels must be interpreted in the context of other clinical information.

Another important consideration is that the majority of DMD patients are treated with corticosteroids (e.g. prednisone and deflazacort), which are generally considered to promote muscle anabolism, again suggestive of a link between ex-myomiR levels and muscle mass

[47,48]. Of note, serum myomiR levels have been reported to be significantly higher in DMD patients receiving daily steroid treatment than in patients treated with a less aggressive intermittent steroid regimen (10 days on, 10 days off) [30], which is important because this may further confound efforts to detect a pharmacodynamic ex-myomiR response to exon skipping therapy [49]. Consequently, ex-myomiR levels may be influenced by therapeutic interventions other than exon skipping, and de-convoluting these responses may prove to be challenging. (The effect of combined exon skipping and glucocorticoid treatment on ex-myomiR levels has yet to be assessed in animal models).

Cellular Mechanisms of MyomiR Release

Understanding the processes which underlie ex-myomiR release is important for accurately interpreting the clinical significance of their altered levels in the circulation. Initially, it was assumed that ex-myomiRs were passively released from damaged fibers that are either undergoing myonecrosis or exhibit 'leakiness' on account of defects in the sarcolemma [29,31,50]. However, several observations from our group, and others, suggest that this explanation is too simplistic. Firstly, alterations in the expression of miRNAs in dystrophic muscle do not necessarily result in a corresponding change in ex-miRNA abundance [39]. For example, expression of miR-1 and miR-133 is decreased in skeletal muscle but highly increased in serum, whereas miR-206 is moderately elevated in *mdx* muscle, and very highly elevated in *mdx* serum [39]. Given that miR-206 is predominantly expressed in immature regenerating fibers in mouse [51], these observations strongly suggest that some ex-myomiRs originate from these fibres. If miRNAs are passively released by non-selective mechanisms (e.g. membrane leakage),

12

then it follows that those miRNAs which are elevated in dystrophic serum might simply reflect the most highly abundant miRNAs in muscle (i.e. as a sampling of myofiber cell contents). While the myomiRs are very highly abundant in skeletal muscle, this is not the case for all miRNAs (e.g. the let-7 family which are highly abundant in muscle and not elevated in *mdx* serum), which strongly suggests that ex-miRNA release might be selective, at least to some extent [40,41]. Secondly, ex-myomiRs have been demonstrated to exhibit dynamic patterns of abundance which mirror pathological events occurring in dystrophic muscles. Specifically, peaks of ex-myomiR levels in *mdx* serum are observed at ~4 weeks of (i.e. the early crisis phase of pathology with when necrosis is widespread [52]) and at ~12 weeks of age (whereby necrosis has largely been overtaken by muscle regeneration [53]). In addition, ex-miR-206 abundance has been found to correlate with muscle tissue expression levels of the myogenic transcription factor *Myog* in *mdx* tibialis anterior [40], consistent with the idea that ex-myomiRs might be used as biomarkers of muscle regenerative status (i.e. repair), and not only of muscle damage. Thirdly, ex-myomiRs have been reported to be elevated in various physiological contexts in the absence of necrosis. Specifically, serum myomiRs have been shown to be elevated during perinatal muscle development in mice, even in the absence of dystrophic pathology [41]. Moreover, progressive release of myomiRs has been demonstrated to be selective during the differentiation of healthy human and mouse myoblasts as a non-muscle specific miRNA of similar cellular abundance, let-7a, was only detected in the media at very low levels [41]. The observations that ex-myomiR concentrations were increased in mouse myogenic cell culture supernatants after treatment with Fibroblast Growth Factor, Tumor Necrosis Factor-α, and Transforming Growth Factor-β) [32], and were repressed by sevoflurane anesthesia in wild-type Wistar rats *in vivo*

[54], demonstrate that there are cellular mechanisms which control ex-miRNA release that can be modulated by external stimuli. Furthermore, studies in immortalized mouse myoblasts derived from *mdx* mice (H-2K-*mdx*) have shown that the absence of dystrophin itself results in disruption of vesicular trafficking and increased protein secretion, independent of myonecrosis [55]. However, whether such phenomena can influence ex-myomiR levels has yet to be demonstrated. Lastly, acute exercise in *mdx* mice results in a biphasic pattern of ex-myomiR release whereby serum myomiR levels are elevated immediately after exercise, and ~5 days later (during the regenerative phase that follows exercise-induced muscle injury) [41]. Taken together, these studies suggest that passive mechanisms are insufficient to completely explain ex-myomiR release, and that regenerating fibers might also contribute to the pool of circulating miRNAs.

In summary, we propose that at least four processes might contribute to ex-myomiR levels: (i) release of cellular contents following myonecrosis, (ii) passive leakage due to impairment of the sarcolemma, (iii) abnormal secretion as a direct consequence of dystrophin deficiency, and (iv) selective release of myomiRs which accompany muscle differentiation and regeneration via mechanisms as yet to be determined (**Figure 1**).

Functional Transfer of miRNAs Between Cells in the Muscle Niche

While the biomarker potential of ex-miRNAs has been extensively studied, their biological significance in the extracellular space is unclear, and subject to ongoing debate [56,57]. Notably, **Extracellular Vesicles** (EVs) (**Box 4**) have been shown to mediate the transfer of mRNA and miRNA between cells, and thereby facilitate intercellular communication by regulating gene

expression in recipient tissues/cells [58]. Given that muscle is considered a secretory organ that communicates with the liver and brain in a contraction-dependent manner (to regulate energy metabolism and endorphin release respectively) [59] it is tempting to speculate that ex-myomiRs may themselves act as '**myokine**' signals that transfer gene regulatory information between organs, or cells within the muscle niche. To this end, several studies have reported cell-to-cell myomiR transfer mediated by EVs in murine **C2C12 myotubes** [60], between activated mouse **satellite cells** and myofibroblasts [61], and between human mesenchymal stem cells and mouse myofibers *in vivo* [62]. An important limitation of these studies is the dependence on sub-optimal methodologies of EV isolation (i.e. ultracentrifugation or **ExoQuick precipitation** - methods that are known to compromise EV purity and integrity) which limits the usefulness of these findings.

EV-mediated myomiR transfer would constitute an elegant means of facilitating cell-to-cell communication within muscle as EV release is a regulated process, encapsulation within EVs would protect miRNAs from serum nuclease digestion, and the protein content of the EV membrane might confer targeting to specific recipient cell types. However, several lines of evidence argue against this proposed biological function of ex-myomiRs. Most importantly, the majority of ex-myomiRs are non-vesicular [40,41] (**Box 4**). While this does not preclude the possibility of EV-mediated transfer between cells, it does raise the question as to whether the number of transferred myomiRs would be sufficient to modulate gene expression in recipient cells. A general problem for the field is that in many EV studies, the quantity of vesicles (~1 µg) applied per cell is often comparable to the total number of EVs present in human blood plasma

[63], and consequently, evidence of functional transfer is of questionable physiological relevance in these contexts. Furthermore, a quantitative analysis of the miRNA content of human exosomes has suggested that most miRNAs are present at very low levels (less than one copy per exosome) [64].

These limitations emphasize that it is important to understand the biological significance of non-vesicular ex-myomiRs. To this end, it has been suggested that the concentration of ex-miRNAs in serum is generally too low to be able to influence gene expression in target cells [65]. However, it is reasonable to assume that ex-miRNA concentrations are much higher in the interstitial fluid of skeletal muscle (relative to the circulation). Consequently, we propose a model whereby differentiating myotubes secrete myomiRs locally in order to activate neighboring satellite cells, and thereby amplify the regenerative response in a feed-forward, paracrine manner [41] (**Figure 2**). We posit that the high concentration of myomiRs in mature/regenerating myofibers (and very low concentration in neighboring quiescent satellite cells [66,67]) implies a directionality to this hypothetical ex-myomiR signaling process, whereby the former transfers gene regulatory information to the latter. In support of this hypothesis, it has been shown that injection of exogenous oligonucleotide myomiR mimics (of miR-1, miR-133, and miR-206) can accelerate muscle regeneration after surgical muscle laceration injury in rats [68]. An alternative hypothesis is that ex-myomiRs might facilitate communication between other cell types in muscle, such as motor neurons, immune cells, myofibroblasts, and fibro/adipogenic progenitors (**Figure 1**). While this proposition has yet to be rigorously tested, C2C12-derived EVs have been shown to enhance neurite outgrowth in the NSC-34 murine a

motor neuron cell line [69], and EV-mediated communication has been demonstrated at the neuromuscular junction in drosophila [70], suggesting that this hypothesis is at least plausible.

While a plethora of studies have described EV-mediated miRNA transfer [57,71], to our knowledge there are no studies that have demonstrated similar effects attributable to non-vesicular ex-miRNAs. The functions, if any, of this non-vesicular majority are therefore unknown at present. One possible scenario would be that non-vesicular myomiRs are continuously secreted, either as part of the natural cycle of cellular miRNA turnover, or to attenuate their functions as gene expression regulators. Notably, non-gene regulatory roles for ex-miRNAs have also been reported (e.g. stimulation of TLR signaling [72]), although there is currently no evidence of this being the case for the myomiRs.

Given the various technical and conceptual problems associated with intercellular ex-miRNA signaling, the issue of whether this phenomenon is truly of biological importance in the context of muscle function and dystrophic pathology remains an open question.

Lessons From miRNA Biomarker Studies in Other Neuromuscular Disorders

Dystrophin acts as an organizing center for the **Dystrophin Associated Protein Complex (DAPC)**, which functions to link the intracellular actin cytoskeleton with the extracellular matrix at the sarcolemma. Consequently, mutations which disrupt other components of the DAPC are causative for a plethora of related muscular dystrophies. Indeed, elevated levels of serum myomiRs have been reported in mouse models of **sarcoglycanopathy** [31,73], **Congenital**

Muscular Dystrophy Type 1A [74], **dysferlinopathy**, and **calpainopathy** [73]. Conversely, elevated serum/plasma myomiR levels have also been reported in neuromuscular disorders with molecular pathologies that are unrelated to DAPC disruption, such as **Myotonic Dystrophy Types I and II** [75,76], **Spinal Muscular Atrophy** [77], and **Amyotrophic Lateral Sclerosis** [78]. Consequently, elevated levels of serum myomiRs are not specific for conditions related to DAPC disruption, but instead may be a consequence of generic muscle pathology. There are several notable exceptions, however, as serum myomiR levels are unchanged in the serum of patients with **Ullrich Congenital Muscular Dystrophy patients** [30] and in the case of a murine model of **Emery-Dreifuss Muscular Dystrophy** [31] (both muscular dystrophies with relatively mild regenerative/degenerative pathologies). In summary, ex-myomiRs are unlikely to be effective at differentiating between DMD and other neuromuscular pathologies, and instead ought to be considered as general candidate biomarkers of muscle turnover [40].

Concluding Remarks

In conclusion, ex-myomiRs may have utility for the assessment of pharmacodynamic responses in exon skipping clinical trials, although an understanding of the relationship between serum levels myomiR levels and assessments of muscle function is currently incomplete (**Box 5**). Evidently, without revisiting outcome measures and developing improved biomarkers for clinical trials, approval of novel DMD therapeutics may remain elusive. However, it is important to note that efficacious treatments are needed in order to validate therapeutic monitoring biomarkers in DMD patients, highlighting that advances in treatment and biomarker discovery are interdependent on one another.

A challenge for future DMD biomarker discovery studies is to identify biomarkers which can inform on the status of distinct pathological processes occurring in dystrophic muscle (e.g. inflammation, fibrosis, adipogenic degeneration, and dilated cardiomyopathy) as these would offer useful additional clinical information. While ex-miRNAs may serve this purpose, other extracellular non-coding RNAs (e.g. PIWI-interacting RNAs, transfer RNA fragments, long non-coding RNAs) represent under-investigated, alternative sources of putative biomarkers. Additionally, non-molecular indications (e.g. timed function tests, pulmonary function, and quality of life measures such as patient/parent/caregiver reported outcomes) should also not be discounted.

Current evidence suggests that multiple factors contribute to ex-myomiR levels including necrosis, muscle regeneration, tissue expression levels, physical activity, and overall muscle mass. An understanding of these factors will be essential for the accurate interpretation of myomiR levels in the serum of DMD patients. Many questions remain regarding the mechanisms of ex-myomiR release, the function of non-vesicular ex-miRNAs, and the extent to which ex-myomiRs participate in cell-to-cell signaling events in the muscle niche (Outstanding Questions and **Box 5**). An exciting possibility is that ex-miRNA-mediated modulation of the regenerative response, or other aspects of dystrophic pathology, might present novel opportunities for therapeutic interventions in DMD.

References

1   Mendell, J.R. and Lloyd-Puryear, M. (2013) Report of MDA muscle disease symposium on newborn screening for Duchenne muscular dystrophy. *Muscle Nerve* 48, 21–26

2   Hoffman, E.P. *et al.* (1987) Dystrophin: The protein product of the duchenne muscular dystrophy locus. *Cell* 51, 919–928

3   Syed, Y.Y. (2016) Eteplirsen: First Global Approval. *Drugs* 76, 1699–1704

4   Dowling, J.J. (2016) Eteplirsen therapy for Duchenne muscular dystrophy: skipping to the front of the line. *Nat Rev Neurol* 12, 675–676

5   Muntoni, F. *et al.* (2017) Response to "Railroading at the FDA." *Nat Biotech* 35, 207–209

6   (2016) FDA Briefing Document, Peripheral and Central Nervous System Drugs Advisory Committee Meeting, January 22, 2016, NDA 206488, Eteplirsen. at <https://www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/drugs/peripheralandcentralnervoussystemdrugsadvisorycommittee/ucm481911.pdf>

7   Wood, M.J.A. (2013) To Skip or Not to Skip: That Is the Question for Duchenne Muscular Dystrophy. *Mol Ther* 21, 2131–2132

8   Lu, Q. *et al.* (2014) What Can We Learn From Clinical Trials of Exon Skipping for DMD? *Mol Ther Nucleic Acids* 3, e152

9   Aartsma-Rus, A. *et al.* (2014) Biomarkers and surrogate endpoints in Duchenne: Meeting report. *Neuromuscular Disorders* 24, 743–745

10   Aartsma-Rus, A. and Spitali, P. (2015) Circulating Biomarkers for Duchenne Muscular Dystrophy. *J Neuromuscul Dis* 2, S49–S58

11   Mitchell, P.S. *et al.* (2008) Circulating microRNAs as stable blood-based markers for cancer detection. *Proc. Natl. Acad. Sci. U.S.A* 105, 10513–10518

12   Kosaka, N. *et al.* (2013) Trash or Treasure: extracellular microRNAs and cell-to-cell communication. *Front Genet* 4, 173

13   Kinali, M. *et al.* (2009) Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study. *Lancet Neurol* 8, 918–928

14   Cirak, S. *et al.* (2011) Exon skipping and dystrophin restoration in patients with Duchenne muscular dystrophy after systemic phosphorodiamidate morpholino oligomer treatment: an open-label, phase 2, dose-escalation study. *Lancet* 378, 595–605

15   Voit, T. *et al.* (2014) Safety and efficacy of drisapersen for the treatment of Duchenne muscular dystrophy (DEMAND II): an exploratory, randomised, placebo-controlled phase 2 study. *The Lancet Neurology* 13, 987–996

16   Mendell, J.R. *et al.* (2013) Eteplirsen for the treatment of Duchenne muscular dystrophy. *Ann. Neurol.* 74, 637–647

17   Mendell, J.R. (2017) Reply. *Ann Neurol.* 81, 164–165

18   Mendell, J.R. *et al.* (2016) Longitudinal effect of eteplirsen versus historical control on ambulation in Duchenne muscular dystrophy. *Ann Neurol* 79, 257–271

19   Zatz, M. *et al.* (1991) Serum creatine-kinase (CK) and pyruvate-kinase (PK) activities in Duchenne (DMD) as compared with Becker (BMD) muscular dystrophy. *J. Neurol. Sci.* 102, 190–196

20   Burch, P.M. *et al.* (2015) Muscle-Derived Proteins as Serum Biomarkers for Monitoring Disease Progression in Three Forms of Muscular Dystrophy. *J Neuromuscul Dis* 2, 241–255

21   Kim, H.K. *et al.* (2010) T2 mapping in Duchenne muscular dystrophy: distribution of disease activity and correlation with clinical assessments. *Radiology* 255, 899–908

22   Hathout, Y. *et al.* (2016) Clinical utility of serum biomarkers in Duchenne muscular dystrophy. *Clin Proteomics* 13, 9

23   Mraz, M. *et al.* (2009) MicroRNA isolation and stability in stored RNA samples. *Biochem. Biophys. Res. Commun.* 390, 1–4

24   Roberts, T.C. *et al.* (2014) Assessment of RT-qPCR Normalization Strategies for Accurate Quantification of Extracellular microRNAs in Murine Serum. *PLoS ONE* 9, e89237

25   Roberts, T.C. *et al.* (2014) Detection and quantification of extracellular microRNAs in murine biofluids. *Biol Proced Online* 16, 5

26   Ray, S. *et al.* (2011) Proteomic technologies for the identification of disease biomarkers in serum: advances and challenges ahead. *Proteomics* 11, 2139–2161

27   Coenen-Stass, A.M.L. *et al.* (2015) Identification of novel, therapy-responsive protein biomarkers in a mouse model of Duchenne muscular dystrophy by aptamer-based serum proteomics. *Sci Rep* 5, 17014

28   Laterza, O.F. *et al.* (2009) Plasma MicroRNAs as Sensitive and Specific Biomarkers of Tissue Injury. *Clinical Chemistry* 55, 1977–1983

29   Cacchiarelli, D. *et al.* (2011) miRNAs as serum biomarkers for Duchenne muscular dystrophy. *EMBO Mol Med* 3, 258–265

30   Zaharieva, I.T. *et al.* (2013) Dystromirs as serum biomarkers for monitoring the disease severity in duchenne muscular dystrophy. *PLoS ONE* 8, e80263

31   Vignier, N. *et al.* (2013) Distinctive serum miRNA profile in mouse models of striated muscular pathologies. *PLoS ONE* 8, e55281

32   Li, X. *et al.* (2014) Circulating Muscle-specific miRNAs in Duchenne Muscular Dystrophy Patients. *Mol Ther Nucleic Acids* 3, e177

33   Hu, J. *et al.* (2014) Serum miR-206 and other muscle-specific microRNAs as non-invasive biomarkers for Duchenne muscular dystrophy. *J. Neurochem.* 129, 877–883

34   Matsuzaka, Y. *et al.* (2014) Three novel serum biomarkers, miR-1, miR-133a, and miR-206 for Limb-girdle muscular dystrophy, Facioscapulohumeral muscular dystrophy, and Becker muscular dystrophy. *Environ Health Prev Med* 19, 452–458

35   Jeanson-Leh, L. *et al.* (2014) Serum profiling identifies novel muscle miRNA and cardiomyopathy-related miRNA biomarkers in Golden Retriever muscular dystrophy dogs and Duchenne muscular dystrophy patients. *Am. J. Pathol.* 184, 2885–2898

36   Llano-Diez, M. *et al.* (2017) Digital PCR quantification of miR-30c and miR-181a as serum biomarkers for Duchenne muscular dystrophy. *Neuromuscul. Disord.* 27, 15–23

37   Anaya-Segura, M.A. *et al.* (2016) Serum Levels of MicroRNA-206 and Novel Mini-STR Assays for Carrier Detection in Duchenne Muscular Dystrophy. *Int J Mol Sci* 17,

38   Mizuno, H. *et al.* (2011) Identification of muscle-specific microRNAs in serum of muscular dystrophy animal models: promising novel blood-based markers for muscular dystrophy. *PLoS ONE* 6, e18388

39	Roberts, T.C. *et al.* (2012) Expression Analysis in Multiple Muscle Groups and Serum Reveals Complexity in the MicroRNA Transcriptome of the mdx Mouse with Implications for Therapy. *Molecular Therapy — Nucleic Acids* 1, e39

40	Roberts, T.C. *et al.* (2013) Extracellular microRNAs are dynamic non-vesicular biomarkers of muscle turnover. *Nucl. Acids Res.* 41, 9500–9513

41	Coenen-Stass, A.M.L. *et al.* (2016) Selective release of muscle-specific, extracellular microRNAs during myogenic differentiation. *Hum. Mol. Genet.* 25, 3960–3974

42	Goyenvalle, A. *et al.* (2012) Rescue of severely affected dystrophin/utrophin-deficient mice through scAAV-U7snRNA-mediated exon skipping. *Hum. Mol. Genet.* 21, 2559–2571

43	McDonald, C.M. *et al.* (2010) The 6-minute walk test in Duchenne/Becker muscular dystrophy: longitudinal observations. *Muscle Nerve* 42, 966–974

44	Gomes, C.P.C. *et al.* (2014) Circulating miR-1, miR-133a, and miR-206 levels are increased after a half-marathon run. *Biomarkers* 19, 585–589

45	Godfrey, C. *et al.* (2015) How much dystrophin is enough: the physiological consequences of different levels of dystrophin in the mdx mouse. *Hum. Mol. Genet.* 24, 4225–4237

46	(2016) Sarepta Therapeutics. Peripheral and Central Nervous System Drugs Advisory Committee eteplirsen briefing document, NDA 206488 (FDA). at <http://www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/drugs/peripheralandcentralnervoussystemdrugsadvisorycommittee/ucm497064.pdf>

47	Ricotti, V. *et al.* (2013) Long-term benefits and adverse effects of intermittent versus daily glucocorticoids in boys with Duchenne muscular dystrophy. *J. Neurol. Neurosurg. Psychiatr.* 84, 698–705

48	Matthews, E. *et al.* (2016) Corticosteroids for the treatment of Duchenne muscular dystrophy. *Cochrane Database Syst Rev* DOI: 10.1002/14651858.CD003725.pub4

49	Merlini, L. and Sabatelli, P. (2015) Improving clinical trial design for Duchenne muscular dystrophy. *BMC Neurol* 15, 153

50	Govoni, A. *et al.* Ongoing therapeutic trials and outcome measures for Duchenne muscular dystrophy. *Cell. Mol. Life Sci.* DOI: 10.1007/s00018-013-1396-z

51	Yuasa, K. *et al.* (2008) MicroRNA-206 is highly expressed in newly formed muscle fibers: implications regarding potential for muscle regeneration and maturation in muscular dystrophy. *Cell Struct. Funct* 33, 163–169

52	Collins, C.A. and Morgan, J.E. (2003) Duchenne's muscular dystrophy: animal models used to investigate pathogenesis and develop therapeutic strategies. *Int J Exp Pathol* 84, 165–172

53	Villalta, S.A. *et al.* (2009) Shifts in macrophage phenotypes and macrophage competition for arginine metabolism affect the severity of muscle pathology in muscular dystrophy. *Hum. Mol. Genet.* 18, 482–496

54	Takeuchi, J. *et al.* (2014) Sevoflurane anesthesia persistently downregulates muscle-specific microRNAs in rat plasma. *Int. J. Mol. Med.* 34, 291–298

55	Duguez, S. *et al.* (2013) Dystrophin deficiency leads to disturbance of LAMP1-vesicle-associated protein secretion. *Cell. Mol. Life Sci.* DOI: 10.1007/s00018-012-1248-2

56	Turchinovich, A. *et al.* (2016) Extracellular miRNA: A Collision of Two Paradigms. *Trends Biochem. Sci.* 41, 883–892

57   Coenen-Stass, A.M.L. *et al.* (2015) Extracellular microRNAs in Membrane Vesicles and Non-vesicular Carriers. *EXS* 106, 31–53

58   Valadi, H. *et al.* (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659

59   Pedersen, B.K. (2013) Muscle as a secretory organ. *Compr Physiol* 3, 1337–1362

60   Forterre, A. *et al.* (2014) Myotube-derived exosomal miRNAs downregulate Sirtuin1 in myoblasts during muscle cell differentiation. *Cell Cycle* 13, 78–89

61   Fry, C.S. *et al.* (2017) Myogenic Progenitor Cells Control Extracellular Matrix Production by Fibroblasts during Skeletal Muscle Hypertrophy. *Cell Stem Cell* 20, 56–69

62   Nakamura, Y. *et al.* (2015) Mesenchymal-stem-cell-derived exosomes accelerate skeletal muscle regeneration. *FEBS Lett.* 589, 1257–1265

63   Sverdlov, E.D. (2012) Amedeo Avogadro's cry: what is 1 µg of exosomes? *Bioessays* 34, 873–875

64   Chevillet, J.R. *et al.* (2014) Quantitative and stoichiometric analysis of the microRNA content of exosomes. *Proc. Natl. Acad. Sci. U.S.A.* 111, 14888–14893

65   Williams, Z. *et al.* (2013) Comprehensive profiling of circulating microRNA via small RNA sequencing of cDNA libraries reveals biomarker potential and limitations. *Proc Natl Acad Sci U S A* 110, 4255–4260

66   Chen, J.-F. *et al.* (2010) microRNA-1 and microRNA-206 regulate skeletal muscle satellite cell proliferation and differentiation by repressing Pax7. *J Cell Biol* 190, 867–879

67   Cheung, T.H. *et al.* (2012) Maintenance of muscle stem-cell quiescence by microRNA-489. *Nature* 482, 524–528

68   Nakasa, T. *et al.* (2010) Acceleration of muscle regeneration by local injection of muscle-specific microRNAs in rat skeletal muscle injury model. *J. Cell. Mol. Med.* 14, 2495–2505

69   Madison, R.D. *et al.* (2014) Extracellular vesicles from a muscle cell line (C2C12) enhance cell survival and neurite outgrowth of a motor neuron cell line (NSC-34). *J Extracell Vesicles* 3,

70   Korkut, C. *et al.* (2013) Regulation of postsynaptic retrograde signaling by presynaptic exosome release. *Neuron* 77, 1039–1046

71   Zhang, J. *et al.* (2015) Exosome and exosomal microRNA: trafficking, sorting, and function. *Genomics Proteomics Bioinformatics* 13, 17–24

72   Lehmann, S.M. *et al.* (2012) An unconventional role for miRNA: let-7 activates Toll-like receptor 7 and causes neurodegeneration. *Nat. Neurosci.* 15, 827–835

73   Israeli, D. *et al.* (2016) Circulating miRNAs are generic and versatile therapeutic monitoring biomarkers in muscular dystrophies. *Sci Rep* 6, 28097

74   Holmberg, J. *et al.* (2014) Laminin α2 Chain-Deficiency is Associated with microRNA Deregulation in Skeletal Muscle and Plasma. *Front Aging Neurosci* 6, 155

75   Koutsoulidou, A. *et al.* (2015) Elevated Muscle-Specific miRNAs in Serum of Myotonic Dystrophy Patients Relate to Muscle Disease Progress. *PLoS One* 10,

76   Perfetti, A. *et al.* (2016) Validation of plasma microRNAs as biomarkers for myotonic dystrophy type 1. *Sci Rep* 6,

77   Catapano, F. *et al.* (2016) Altered Levels of MicroRNA-9, -206, and -132 in Spinal Muscular Atrophy and Their Response to Antisense Oligonucleotide Therapy. *Mol Ther Nucleic Acids* 5, e331

78   Toivonen, J.M. *et al.* (2014) MicroRNA-206: A Potential Circulating Biomarker Candidate for Amyotrophic Lateral Sclerosis. *PLoS ONE* 9, e89065

79   Petrof, B.J. *et al.* (1993) Dystrophin protects the sarcolemma from stresses developed during muscle contraction. *Proc. Natl. Acad. Sci. U.S.A.* 90, 3710–3714

80   Almada, A.E. and Wagers, A.J. (2016) Molecular circuitry of stem cell fate in skeletal muscle regeneration, ageing and disease. *Nat. Rev. Mol. Cell Biol.* 17, 267–279

81   Carnwath, J.W. and Shotton, D.M. (1987) Muscular dystrophy in the mdx mouse: histopathology of the soleus and extensor digitorum longus muscles. *J. Neurol. Sci.* 80, 39–54

82   Chen, Y.-W. *et al.* (2005) Early onset of inflammation and later involvement of TGFbeta in Duchenne muscular dystrophy. *Neurology* 65, 826–834

83   Porter, J.D. *et al.* (2002) A chronic inflammatory response dominates the skeletal muscle molecular signature in dystrophin-deficient mdx mice. *Hum. Mol. Genet* 11, 263–272

84   Rodeheffer, M.S. (2010) Tipping the scale: muscle versus fat. *Nat. Cell Biol.* 12, 102–104

85   Bushby, K. *et al.* (2010) Diagnosis and management of Duchenne muscular dystrophy, part 1: diagnosis, and pharmacological and psychosocial management. *Lancet Neurol* 9, 77–93

86   Hufton, M. and Roper, H. (2017) Variations in Duchenne muscular dystrophy course in a multi-ethnic UK population: potential influence of socio-economic factors. *Dev Med Child Neurol* 59, 837–842

87   Koenig, M. *et al.* (1987) Complete cloning of the Duchenne muscular dystrophy (DMD) cDNA and preliminary genomic organization of the DMD gene in normal and affected individuals. *Cell* 50, 509–517

88   Monaco, A.P. *et al.* (1988) An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics* 2, 90–95

89   England, S.B. *et al.* (1990) Very mild muscular dystrophy associated with the deletion of 46% of dystrophin. *Nature* 343, 180–182

90   Matsumura, K. *et al.* (1994) Immunohistochemical analysis of dystrophin-associated proteins in Becker/Duchenne muscular dystrophy with huge in-frame deletions in the NH2-terminal and rod domains of dystrophin. *J. Clin. Invest.* 93, 99–105

91   Aartsma-Rus, A. *et al.* (2009) Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. *Hum. Mutat.* 30, 293–299

92   Filipowicz, W. *et al.* (2005) Post-transcriptional gene silencing by siRNAs and miRNAs. *Curr. Opin. Struct. Biol* 15, 331–341

93   Lee, Y. *et al.* (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature* 425, 415–419

94   Bernstein, E. *et al.* (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* 409, 363–366

95   Roberts, T.C. (2015) The microRNA Machinery. *Adv. Exp. Med. Biol.* 887, 15–30

96   Chen, J.-F. *et al.* (2006) The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. *Nat. Genet.* 38, 228–233

97   Ikeda, S. *et al.* (2009) MicroRNA-1 negatively regulates expression of the hypertrophy-associated calmodulin and Mef2a genes. *Mol. Cell. Biol.* 29, 2193–2204

98   Greco, S. *et al.* (2009) Common micro-RNA signature in skeletal muscle damage and regeneration induced by Duchenne muscular dystrophy and acute ischemia. *FASEB J* 23, 3335–3346

99   Eisenberg, I. *et al.* (2007) Distinctive patterns of microRNA expression in primary muscular disorders. *Proc. Natl. Acad. Sci. U.S.A* 104, 17016–17021

100   Roberts, T.C. *et al.* (2015) Multi-level omics analysis in a murine model of dystrophin loss and therapeutic restoration. *Hum. Mol. Genet.* 24, 6756–6768

101   Roberts, T.C. and Wood, M.J.A. (2013) Therapeutic targeting of non-coding RNAs. *Essays in Biochemistry* 54, 127–145

102   Zanotti, S. *et al.* (2015) Opposing roles of miR-21 and miR-29 in the progression of fibrosis in Duchenne muscular dystrophy. *Biochim. Biophys. Acta* 1852, 1451–1464

103   Cacchiarelli, D. *et al.* (2011) miR-31 modulates dystrophin expression: new implications for Duchenne muscular dystrophy therapy. *EMBO Rep.* 12, 136–141

104   Wang, L. *et al.* (2012) Loss of miR-29 in Myoblasts Contributes to Dystrophic Muscle Pathogenesis. *Molecular Therapy* 20, 1222–1233

105   Liu, N. *et al.* (2012) microRNA-206 promotes skeletal muscle regeneration and delays progression of Duchenne muscular dystrophy in mice. *J. Clin. Invest.* 122, 2054–2065

106   Arroyo, J.D. *et al.* (2011) Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5003–5008

107   Turchinovich, A. *et al.* (2011) Characterization of extracellular circulating microRNA. *Nucleic Acids Res.* 39, 7223–7233

108   Turchinovich, A. and Burwinkel, B. (2012) Distinct AGO1 and AGO2 associated miRNA profiles in human cells and blood plasma. *RNA Biol* 9, 1066–75

109   Vickers, K.C. *et al.* (2011) MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat. Cell Biol.* 13, 423–433

Acknowledgments

Study Design	miRNAs	Notable Findings	Citation
DMD (*n*=26) Age 1-16 BMD (*n*=5) Healthy (*n*=7)	miR-1 miR-133 miR-206	Intermediate myomiR levels in BMD serum. Inverse correlations between ex-myomiR levels and age, NSAA scores (age 3-6). (Correlations were stronger than for serum CK).	[29]
DMD (*n*=44) BMD (*n*=5) Healthy (*n*=14)	miR-1 miR-133 miR-206	Intermediate ex-myomiR levels in BMD patients. Ex-myomiRs higher in ambulant patients. Ex-myomiR levels higher in patients on daily steroid regimen. No correlation with NSAA (age 4-13). No correlation with cardiomyopathy. Positive correlation with FVC. Slight decrease in ex-myomiRs with age.	[30]
DMD (*n*=5) Age 5=7	miR-1 miR-133 miR-206 miR-378 miR-378* miR-31	NA	[31]
DMD (*n*=52) BMD (*n*=15) Healthy (*n*=23) Age 1-14	miR-1 miR-133 miR-206 miR-208a miR-208b miR-499	Intermediate miR-206 and miR-499 levels in BMD serum. No correlation between ex-miRNA levels and age overall. Positive correlation in a subset of younger patients (age 2-6).	[32]
DMD (*n*=39) Ambulant Age 4-12 Steroid naïve Healthy (*n*=36)	miR-1 miR-133 miR-206	No correlation between ex-myomiR levels and age. Inverse correlations between ex-myomiR levels and muscle strength, function, and quality of life. (opposite the case for CK)	[33]
DMD (*n*=12) Considered as two different groups: Age 5-18 and age 27-31 BMD (*n*=4)	miR-1 miR-133 miR-206	NA	[34]
DMD (*n*=5) Healthy (*n*=3)	miR-499 miR-208b miR-95	No simple correlation between ex-miRNA levels and cardiac function.	[35]
DMD (*n*=21) Age 2-14 BMD (*n*=5 Age 9-15 Healthy (*n*=22) Age 2-14	miR-30c miR-181	Ex-miRNA levels not significantly different between DMD and BMD patients. Ex-miRNA levels were not correlated with age or affected by corticosteroid use.	[36]

Table S1

Summary of Extracellular MicroRNA Studies in DMD and BMD Patients.

Key study design details are shown for all of the major ex-miRNA studies in DMD and BMD patients. All studies analyzed ex-miRNA levels in serum with the exception of the Vignier et al., and Jeanson-Leh et al., which analyzed plasma [31,35]. Sample sizes and patient age ranges are included where information is available. FVC, Forced Vital Capacity. NSAA, North Star Ambulatory Assessment. NA, Not Applicable.

Box 1: Duchenne Muscular Dystrophy Pathology

DMD (incidence: 1:5,000 live male births [1]) is caused by loss of function mutations in the gene encoding the dystrophin protein (human: *DMD*, mouse: *Dmd*) on the X chromosome [2]. Absence of dystrophin sensitizes myofibers to contraction-induced damage [79], leading to chronic cycles of myonecrosis and compensatory regeneration mediated by the muscle satellite (stem) cell pool [80]. Dystrophic muscle is characterized by the presence of necrotic foci, regenerating fibers, and persistent immune cell infiltration [81–83]. Over time, the quality of dystrophic muscle declines on account of extensive fibrosis and deposition of adipose tissue [84]. Typically, patients present with muscle weakness by age 3, lose ambulation around age 9-12, and die from cardiorespiratory failure in the second or third decade of life [2,85,86].

Box 2: Exon Skipping Therapy for DMD

The *DMD* gene consists of 79 exons, many of which code for redundant structural domains that are dispensable for dystrophin function [87]. As a result, *DMD* mutations which do not disrupt the translation reading frame (i.e. in-frame exon deletions) lead to the production of a truncated,

but partially functional dystrophin protein. Such mutations are causative of the much less severe dystrophinopathy, Becker Muscular Dystrophy (BMD), with some BMD patients being effectively asymptomatic [88–90]. DMD is thus amenable to exon skipping (also called splice correction) therapy whereby antisense oligonucleotides are used to induce alternative splicing events which exclude one or more exons from the dystrophin pre-mRNA. The goal of exon skipping is therefore to convert the DMD phenotype into a milder BMD-like phenotype, and thereby reduce disease severity [91].

Box 3: Contribution of MicroRNAs to Muscle Biology and Dystrophic Pathophysiology

Microribonucleic acids (microRNAs, miRNAs, miRs) are short (~22 nucleotide), single-stranded, genome-encoded RNA molecules. The primary function of miRNAs appears to be the execution of post-transcriptional regulation of gene expression [92]. miRNAs are generated by the cleavage of precursor hairpins in two sequential processing reactions by the RNase III enzymes DROSHA and DICER1 [93–95]. The mature miRNA sequence then acts to guide the ribonucleoprotein complex RISC (RNA-Induced Silencing Complex), the catalytic component of which is an Argonaute protein (typically AGO2), to target mRNAs. Two families of closely related miRNAs are critical regulators of myogenesis; miR-1 and miR-206 which differ by four nucleotides, and miR-133a and miR-133b which differ by only a single nucleotide. These miRNAs are highly enriched in human/murine skeletal muscle (and miR-1 and miR-133 are also present in cardiac muscle) and so are collectively referred to as the classical myomiRs. These miRNAs control muscle homeostasis by coordinating satellite cell/myoblast proliferation and differentiation [66,96,97]. Furthermore, specific miRNAs are differentially expressed in

dystrophic muscle [39,98–100], where they contribute to disease processes and are potential targets for therapeutic intervention using anti-miRNA oligonucleotides or synthetic miRNA mimics [101]. For example, inhibition of miR-21 and miR-31 in the *mdx* mouse has been shown to suppress fibrosis and increase dystrophin expression respectively [102,103]. Conversely, administration of synthetic miR-29 and miR-206 mimics has been shown to impede fibrosis and promote muscle regeneration respectively, also in the *mdx* mouse [104,105]. The measurement of muscle miRNA expression also has biomarker potential by informing on pathophysiological processes occurring in dystrophic muscle (e.g. miR-21 and miR-29 in the case of fibrosis). However, these analyses necessitate muscle biopsy, and so biofluid analysis is more preferable for DMD patients.

Box 4. Extracellular MyomiRs Primarily Exist in Non-Vesicular Protein Complexes

A seminal study, hypothesized that the stability of ex-miRNAs in nuclease-rich biofluids such as human plasma could be accounted for by their encapsulation within **Extracellular Vesicles** (EVs) [11]. Indeed, miRNAs are present in EVS [58], and several groups have postulated similar EV-based explanations for the stability of myomiRs in the circulation of DMD patients and dystrophic animals [29,33,38]. However, it has since been shown that the majority of ex-miRNAs in human serum/plasma reside outside EVs and are instead associated with RNA-binding proteins (such as AGO1 and AGO2) [106–108], and with High-Density Lipoprotein (HDL) [109]. To expand these findings in these specific context of DMD, our group analyzed vesicular and non-vesicular RNAs from dystrophic serum, and determined that ex-myomiRs were ~80-99% non-vesicular, as evidenced from multiple physical separation methodologies (i.e.

ultracentrifugation, ultrafiltration, and size exclusion liquid chromatography) [40,41]. Furthermore, serum myomiRs were rapidly degraded after treatment with Proteinase K, and immunoprecipitation assays demonstrated myomiR complex formation with AGO2 and Apolipoprotein A1 (APOA1) [40] supporting the notion that the former might be stabilized by protein/lipoprotein complexes. However, to date, a mechanism for the export of non-vesicular ex-miRNA carriers has yet to be described.

Box 5. Clinician's Corner

- Recent controversy regarding the approval of the exon skipping drug eteplirsen has highlighted the need of appropriate biomarkers and outcome measurements in DMD clinical trials.

- Muscle-enriched microRNAs (the myomiRs; miR-1, miR-133, and miR-206) appear to be promising DMD serum biomarkers which have been shown to be effective in discriminating healthy vs dystrophic individuals. However, these biomarkers are not specific for DMD and are similarly elevated in the case of other neuromuscular disorders.

- Serum myomiRs have exhibited a pronounced response to exon skipping in preclinical animal models, suggesting that they might have utility as pharmacodynamic biomarkers in future DMD clinical trials.

- Serum myomiR levels change substantially as a consequence of aging, DMD disease progression, and physical activity. As a result, extracellular myomiR levels must be interpreted in the context of other clinical information.

31
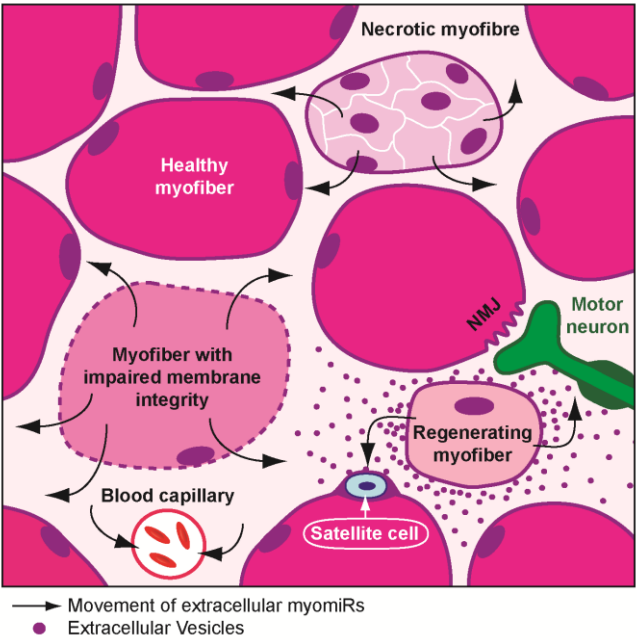
Figure 1

Proposed Mechanisms of ex-myomiR release in the dystrophic muscle niche.

Cartoon of a skeletal muscle transverse section illustrating both normal and dystrophic features. Normal myofibers are depicted with peripheral myonuclei. A degenerating, necrotic myofiber with infiltrating immune cells releases its cellular content (including ex-myomiRs) as a consequence of cell death. Absence of dystrophin leads to impairment of sarcolemma function and sensitizes myofibers to contraction-induced damage. Changes in the permeability of the sarcolemma as a consequence of mechanical stress and downstream molecular consequences of dystrophin loss (i.e. impaired membrane integrity, and disrupted calcium homeostasis) result in ex-myomiR release. A regenerating myofiber is depicted with a centrally located nucleus may also release ex-myomiRs in a regulated and selective manner (e.g. in extracellular vesicles). Such an active release mechanism may serve a signaling function via transfer of miRNAs to resident mononuclear cells in the muscle niche (i.e. satellite cells), or alternatively to motor neurons

which make contact with myofibers via the Neuromuscular Junction (NMJ). Ex-myomiRs which are not taken up by cells in the interstitium may enter the circulation via blood capillaries. (Not to scale, spaces between myofibers have been exaggerated for clarity).
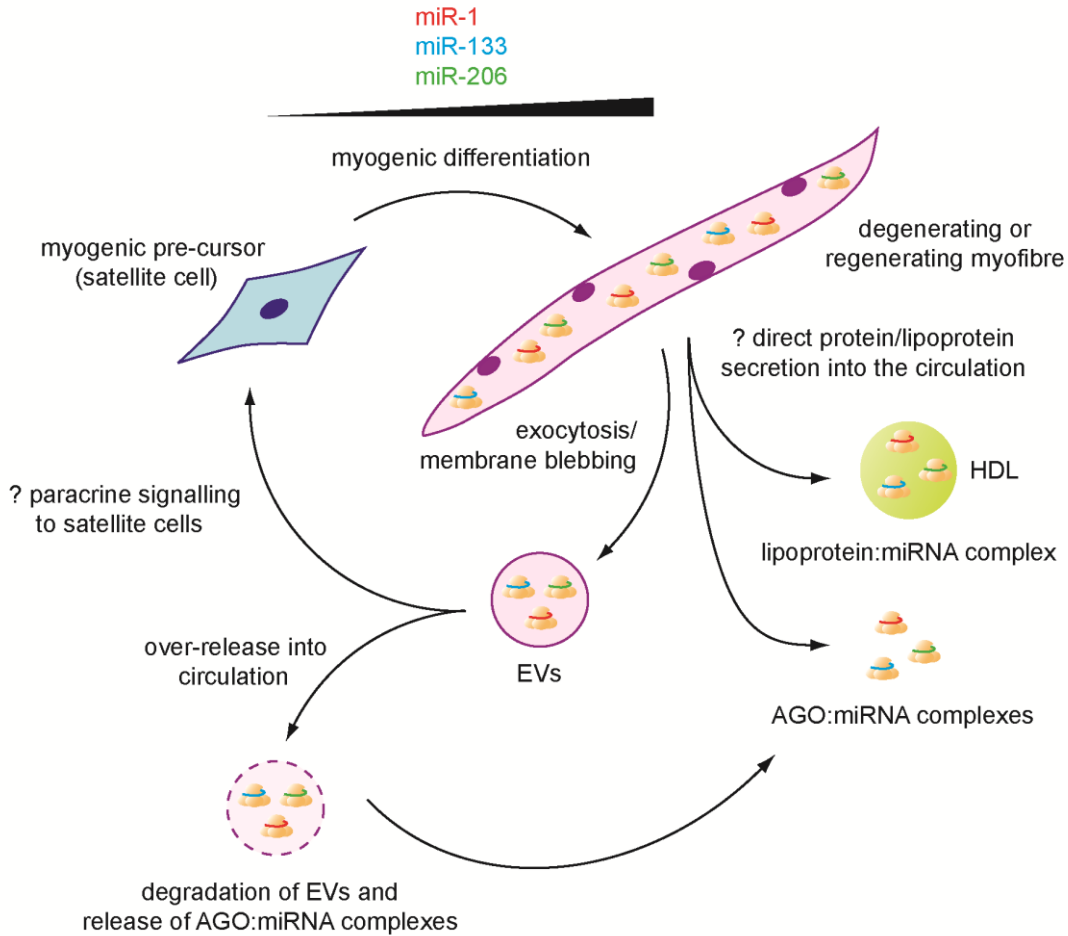
Figure 2

Hypothetical model of extracellular myomiR-mediated feed-forward paracrine signaling.

The myomiRs miR-1, miR-133 and miR-206 (indicated in red, blue and green respectively) are progressively up-regulated during myogenic differentiation as pre-cursor cells (e.g. myoblasts in culture or satellite cells *in vivo*), which initially do not express myomiRs, fuse to form myotubes and myofibers, which then express very high levels of myomiRs. In dystrophic muscle, myofibers release ex-myomiRs into the interstitium as a consequence of fiber damage or concomitant with regeneration. Ex-myomiRs may be secreted within Extracellular Vesicles, EVs (e.g. in exosomes or microvesicles following exocytosis and membrane blebbing respectively),

or released in protein/lipoprotein complexes via unknown mechanisms. Ex-myomiRs may be taken up by myogenic pre-cursor cells in a paracrine manner and thereby promote further differentiation. This feed-forward mechanism would serve to amplify the regenerative response to dystrophic pathology. EVs that escape uptake within the muscle interstitium enter the circulation (i.e. over-release). Subsequently, AGO:miRNA complexes may be liberated as a consequence of EV turnover. AGO, Argonaute protein. HDL, High-Density Lipoprotein.

Glossary

6 Minute Walk Test

The 6 Minute Walk Test (6MWT) is a clinical assessment of the distance (in metres) that an individual can walk within 6 minutes (i.e. 6 minute walk distance). This test is typically used to assess the status of the heart, lungs, and circulation, and overall function of the musculature.

Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a late-onset neurodegenerative disease affecting upper and lower motor neurons leading to a fast-progressing muscular atrophy. The majority of ALS cases are sporadic and the underlying genetic mutations are complex and as yet incompletely understood.

Antisense Oligonucleotide-mediated Exon Skipping

Antisense oligonucleotides ae nucleic acid drugs which bind to their corresponding sense target RNA transcripts by complementary Watson-Crick base pairing. Exon skipping is a molecular therapy approach which aims to alter pre-mRNA splicing with antisense oligonucleotides so as to restore the translation reading frame of a mutation containing transcript. Exon skipping can be induced by masking splicing signals in target intronic or exonic sequences and promoting the exclusion of the targeted exon(s) from the mature mRNA transcript.

Biofluids

Biofluids are biological fluids which include serum, plasma, saliva, urine, cerebrospinal fluid, and interstitial fluid.

Becker Muscular Dystrophy

Becker Muscular Dystrophy (BMD) is the milder allelic form of DMD, characterised by late disease onset and with patients usually remaining ambulant until middle age or beyond. BMD causing mutations are typically in-frame, resulting in the presence an internally truncated, but largely functional dystrophin protein.

Calpainopathy

Mutations in the Calpain 3 (CAPN3) protein can result in calpainopathy, an autosomal recessive muscle-wasting condition that is also referred to as Limb-Girdle Muscular Dystrophy 2A (LGMD2A).

Congenital Muscular Dystrophy Type 1A

Congenital Muscular Dystrophy Type 1A (MDC1A) is an inherited muscle-wasting disorder caused by mutations in the *LAMA2* (Laminin α2) gene.

CXMD$_J$

A canine model of DMD (Canine X-linked Muscular Dystrophy in Japan). Canine X-linked muscular dystrophy describes a canine strain with a splice site mutation in the dystrophin gene, developed in Japan to serve as a large animal model for Duchenne Muscular Dystrophy.

C2C12 myotubes

C2C12 cells are a murine myoblast cell line originally generated from a thigh muscle of a C3H mice mouse after crush injury. In reduced serum conditions, C2C12 myoblast differentiate spontaneously into multinucleated myotubes.

dko mouse

The dystrophin/utrophin Double Knockout (*dko*) mouse model was generated by genetic knockout of the gene coding utrophin, *Utrn*, (a dystrophin paralog) in *mdx* mice. As utrophin is able to compensate for dystrophin to some extent, the *dko* mice exhibit a more severe phenotype than the *mdx* mice with a drastically reduced life expectancy.

Drisapersen

Drisapersen (also known as Kyndrisa) is an experimental exon skipping therapy for DMD developed Prosensa in partnership with GlaxoSmithKline. Prosensa was subsequently acquired by BioMarin Pharmaceutical Inc. who further developed the drug. Drisapesen is an antisense oligonucleotide based on 2′O-methyl phosphorothioate RNA chemistry designed to induce skipping of *DMD* exon 51. Drisapersen is no longer undergoing development after FDA approval was declined, following the failure to meet primary or secondary clinical trial endpoints.

Dysferlinopathy

Dysferlinopathies are adult-onset myopathies caused by mutations in the dysferlin gene with variable clinical presentation. Dysferlin is a transmembrane protein in skeletal muscle which is important for sarcolemma repair.

Dystrophin

Dystrophin is the protein product of the *DMD* locus in humans. Mutations in this gene cause DMD, and the less severe allelic variant BMD. Dystrophin acts as an organizing center for the DAPC, protects myofibers from contractile damage, and is involved in multiple signaling processes.

Dystrophin Associated Protein Complex

The Dystrophin Associated Protein Complex (DAPC) (also called the DystroGlycan Complex, DGC) is a complex of proteins (consisting of dystroglycans, dystrobrevins, syntrophins, sarcoglycans, laminins, dysferlin, neuronal nitric oxide synthase, caveolin 3, and others) which resides at the sarcolemma. The DAPC is believed to act as a link between the extracellular matrix and the actin cytoskeleton. Dystrophin is required for the correct formation of the DAPC. Mutations in the genes encoding many of the DAPC components are causative of various muscular dystrophies.

Emery-Dreifuss Muscular Dystrophy

Emery-Dreifuss Muscular Dystrophy refers to a number of slowly progressing muscular wasting disorders with different genetic causes some of which caused by mutations in the genes encoding the nuclear membrane proteins Emerin (EMD) and Lamin A/C (LMNA).

Eteplirsen

Eteplirsen (trade name Exondys 51) is an experimental exon skipping therapy for DMD developed by Sarepta Therapeutics (previously AVI-BioPharma). Eteplirsen is an antisense oligonucleotide based on PMO chemistry designed to induce skipping of *DMD* exon 51. Eteplirsen currently has FDA accelerated approval status which is conditional on the outcome of an ongoing phase III clinical trial.

ExoQuick precipitation

ExoQuick precipitation is a commercially available methodology for exosome isolation (exosomes are purified by polymer precipitation).

Extracellular RNA

Extracellular RNA includes any RNA species present outside of the cell. Typically, extracellular RNA is protected from degradation by association with EVs, lipoproteins or proteins such as AGO2. miRNAs are one class of extracellular RNA, although there are a plethora of other extracellular RNA types.

Extracellular Vesicles

Extracellular Vesicles (EVs) are a heterogeneous population of lipid-bilayer enclosed vesicles that are released from cells by either fusion of an intermediate endocytic compartment with the cell membrane (exosomes), direct budding from the plasma membrane (microvesicles) or during apoptosis (apoptotic bodies). Depending on their biogenesis, EVs vary in size, lipid composition, and protein/nucleic acid content. Given their ability to transfer their cargos to receipt cells, EVs are of importance for a plethora of biological and pathological processes. Furthermore, EVs may also be harnessed as delivery vehicles for therapeutic cargoes such as transgene DNA, oligonucleotides (such as miRNA mimics) or mRNAs.

GMRD

A canine model of DMD (Golden Retriever Muscular Dystrophy). A naturally occurring splice site mutation in the dystrophin gene in golden retriever dogs is associated with muscular weakness and symptoms reminiscent of Duchenne Muscular Dystrophy.

***mdx* mouse**

The most commonly used murine model of DMD. Dystrophy in the *mdx* mouse is caused by a point mutation in exon 23 of the *Dmd* gene, resulting in a premature termination codon, which arose spontaneously in a C57Bl/10 colony. Affected mice develop muscular dystrophy, albeit with a much milder phenotype compared to DMD patients.

***mdx*^{4cv} mouse**

A murine model of DMD generated by chemically induced-mutation in C57BL/6 mice. Dystrophy in the *mdx^{4cv}* mouse is caused by a point mutation in exon 53, resulting in a premature termination codon.

MicroRNA

MicroRNAs (miRNAs) are a class of small (21-22 nt), non-coding RNAs which primarily regulate gene expression at the post-transcriptional level based on antisense complementary with a messenger RNA (mRNA) target. Depending on the degree of miRNA sequence homology, transcripts are either cleaved, or their translation is repressed.

modified-Intention-To-Treat

Modified-Intention-To-Treat (mITT) analysis is a variation on the Intention-To-Treat (ITT) methodology used in the reporting of randomized controlled clinical trial data. ITT is the gold standard, whereby all participants are analyzed regardless of whether they were correctly treated according to the trial protocols, or if they withdrew from the trial. mITT deviates from this methodology by analyzing a subset of trial participants and excluding others. There may be scientifically valid reasons for utilizing the mITT approach, although it is more prone to bias than ITT.

Myokine

Myokines are secreted factors (e.g. cytokines) released from muscle which act as autocrine, paracrine, or endocrine signaling molecules.

Myotonic Dystrophy Types I and II

Myotonic dystrophy (MD) is the most commonly inherited muscular dystrophy in adults. Both MD type I and II are inherited autosomal dominant and arise from micro-satellite repeat expansions in either the Dystrophia Myotonica Protein Kinase (DMPK) or Cellular Nucleic acid-Binding Protein (CNBP) respectively.

muscle-enriched microRNA (myomiR)

The myomiRs are a subset of miRNAs that are highly enriched in muscle and have been implicated in normal muscle function. Here we use the term myomiR to refer specifically to the classical myomiRs; miR-1, miR-133, and miR-206.

North Star Ambulatory Assessment

The North Star Ambulatory Assessment (NSAA) is a series of exercises which are used to assess muscle function in DMD patients.

Phosphorodiamidate Morpholino Oligomer

Phosphorodiamidate Morpholino Oligomers (PMO) are synthetic nucleic acid used for exon skipping drugs (i.e. antisense oligonculeotides). They are designed to mimic the natural structure of RNA but with improved stability. PMO consists of a morpholine ring instead of ribose and has a neutrally charged backbone that allows for covalent conjugation to other moieties (such as cell-penetrating peptides).

Sarcoglycanopathy

Sarcoglycanopathy refers to a myopathy caused by mutation in one of the four sarcoglycan genes (*SGCA*, *SGCB*, *SGCG*, or *SGCD*). Sarcoglycan proteins are components of the DAPC, and as such are important for the maintenance of myofiber integrity.

Sarcolemmal stability

The sarcolemma is the cell membrane of striated myofibers consisting of the plasma membrane, and its associated polysaccharides and structural proteins. Disruption of proteins which contribute to the stability of the sarcolemma (such as components of the DAPC), or aberrant calcium homeostasis, can result in transient and local increases in membrane permeability, thereby compromising its barrier function. Consequently, small molecules and ions may 'leak' across the sarcolemma.

Satellite cells

Satellite cells are the muscle stem cells which support muscle growth and regeneration.

Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease caused by homozygous deletion of in the Survival of Motor Neuron 1 (SMN1) gene. Death of lower motor neurons leads to severe and progressive muscle-wasting and atrophy.

Surrogate Biomarker

A surrogate biomarker is a measurement that can serve as a substitute for a clinically meaningful endpoint. Such a biomarker should have the capacity to directly predict if a therapy has a positive effect on patient wellbeing.

Ullrich Congenital Muscular Dystrophy

Ullrich Congenital Muscular Dystrophy is an autosomal inherited myopathy caused by mutation in genes coding for the α-subunit of collagen 4 (COL6A1, COL6A2, and COL6A3), important structural components of the extracellular matrix.