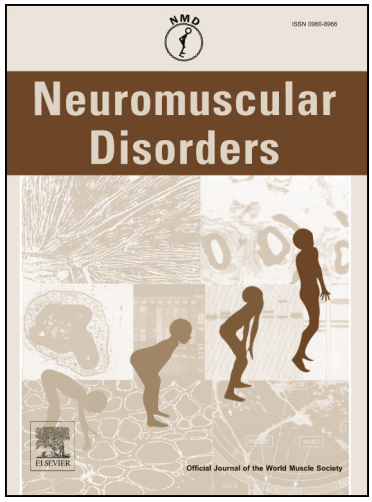


Personal Comment

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PII: S0960-8966(14)00082-0
DOI: http://dx.doi.org/10.1016/j.nmd.2014.03.007
Reference: NMD 2864

To appear in: *Neuromuscular Disorders*

Received Date: 12 January 2014
Revised Date: 11 March 2014
Accepted Date: 13 March 2014

Please cite this article as: Wilton, S.D., Fletcher, S., Flanigan, K.M., Dystrophin as a therapeutic biomarker: are we ignoring data from the past?, *Neuromuscular Disorders* (2014), doi: http://dx.doi.org/10.1016/j.nmd.2014.03.007

**Dystrophin as a therapeutic biomarker: are we ignoring data from the past?**

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Dystrophin is a subsarcolemmal structural protein that provides a link between the actin cytoskeleton and a complex of proteins linked to the extracellular matrix. In the absence of dystrophin, muscle fibres are prone to damage and show altered contractile function and signaling deficits. Duchenne muscular dystrophy (DMD) is caused by mutations in the massive dystrophin (*DMD)* gene that ablate synthesis of the 427kD muscle specific dystrophin isoform. The development of dystrophin-specific antibodies led to the rapid acceptance of dystrophin expression as a diagnostic biomarker, with the lack of the 427kD gene product in muscle signifying DMD [1, 2].

Becker muscular dystrophy (BMD) also arises from dystrophin mutations. However the gene lesions causing BMD are typically whole exon deletions that maintain an open reading frame and allow synthesis of internally truncated dystrophin isoforms retaining some degree of

function [3, 4]. Depending upon the nature and location of the dystrophin gene lesion, BMD individuals remain ambulant until at least 16 years of age, but may be asymptomatic and are either diagnosed late in life, or by chance (for review see [5, 6]).

Antisense oligomer mediated exon skipping is emerging as a promising therapy for this fatal childhood muscle-wasting disease. The most common type of DMD-causing mutation is the frame-shifting deletion of one or more dystrophin exons [3]. Targeted removal of a flanking exon during pre-mRNA processing can re-frame the dystrophin transcript to generate a BMD-like isoform [7-9]. The functionality, and therefore the clinical utility of the induced dystrophin isoform will be determined by the nature and location of the primary gene lesion. Restoration of the reading frame around dystrophin deletions encompassing crucial functional domains (eg β-dystroglycan binding domain) or involving more than 34 exons are unlikely to result in significant clinical improvement [10].

Molecular therapies that aim to restore dystrophin expression have now reached clinical trials, and for the first time, significant functional improvements with unequivocal increased muscle dystrophin expression in DMD has been demonstrated [11]. Nevertheless, questions regarding the relationship of dystrophin expression to functional outcome have been raised in the regulatory evaluation of studies seeking to use dystrophin expression as a biomarker in dystrophin restoring therapies. We believe a review of the literature regarding dystrophin expression provides an essential context for addressing these concerns.

Some clinical studies on dystrophin restoring therapies do not deem it necessary to monitor dystrophin levels, since at this time linear and direct correlations between clinical benefit and induced dystrophin expression have yet to be defined. Different dystrophin isoforms, induced

at different disease stages, and the variable distribution of dystrophin across muscle groups could confound interpretation and validation of therapies, such as antisense oligomer mediated exon skipping. Despite these challenges, restoration of functional dystrophin expression is a direct consequence of exon skipping, and is expected to confer clinical benefits in DMD. Induced dystrophin in muscle should therefore be recognized as an initial primary surrogate endpoint, in combination with clinical endpoints, including the 6 minute walk test and respiratory function. Therapies designed to induce functional dystrophin isoforms should be expected to result in gradual accumulation of the protein over time. Consequently, if exon skipping was to confer a clinical benefit, this should show some correlation with amount and functionality of dystrophin, but this will vary according to mutation, duration of treatment, patient age, genetic background and disease pathology. Although at this time, the amount of dystrophin needed to confer clinical benefits remains uncertain, it is evident that low levels of dystrophin expression can mitigate disease progression [12].

Over twenty years ago, low-level dystrophin expression in DMD patient muscle was reported [12-14], and Nicholson and colleagues hypothesized that dystrophin in DMD muscle could result from exon skipping [12]. Gangopadhyay *et al.* [13] found up to 10-12% of normal dystrophin levels in DMD patients with deletions of exons 3-7, but no evidence of frame-restoring exon skipping. Using a different assay on samples from patients with dystrophin deletions of exons 3-7, Chelly *et al.* identified in-frame dystrophin transcripts with exon 1 spliced to exon 8 and exon 2 joined to exon 10 [15].

Although the Nicholson study [12] included limited numbers of DMD (n=30) and BMD/intermediate muscular dystrophy patients (n=6), dystrophin was detected in all

BMD/intermediate cases, and trace amounts of dystrophin were detected in two thirds of the DMD patient biopsies (18/30 by western blotting and 22/30 by immunostaining). Two non-exclusive dystrophin patterns were observed in DMD muscle: clear staining of a few (<1%) fibres, representing reverant fibres, and weak labeling of about ~25% of fibres. Presumably arising from two distinct mechanisms, these very low levels of dystrophin correlated with a delay in the loss of ambulation by approximately 2 years, compared to those boys with no detectable dystrophin [12]. Similarly, the presence of minor in-frame alternatively spliced mRNAs correlated with expression of truncated dystrophins and a milder than expected phenotype in patients with frame shifting deletions [16].

Although less than 3% of normal dystrophin levels in DMD is insufficient to provide sustained protection for muscle fibres against contracture induced injury, *the fundamental premise of antisense oligomer mediated exon skipping as a therapy is that processing of a DMD gene transcript can be appropriately modified to produce a BMD-like dystrophin isoform*. It is hypothesized that the induced dystrophin isoform will confer functional support and improve muscle fibre integrity, however, the level of induced dystrophin required to provide meaningful clinical benefit is yet to be determined. While the dystrophin transcripts in BMD patients allow correlation of genotype and phenotype, and perhaps provide templates for the more functional dystrophin isoforms, we remain mindful that dystrophin, albeit of variable quality and quantity, is present in BMD muscle from birth. Thus, we should not conclude that dystrophin restitution in DMD patients with established dystrophic pathology will confer comparable benefits to the dystrophins in BMD patients. Despite the limitations of the *mdx* mouse as a model of DMD, animal studies provide some guidelines on the amounts of various dystrophins necessary to protect muscle, and treatments, such as exon skipping can be initiated in adult mice. Isolated muscle studies in PMO treated *mdx* mouse

muscle indicate that a minimum of 20% of dystrophin-positive fibers is necessary to confer resistance to contraction-induced injury, and that a relatively low level of dystrophin expression in muscle fibers may have significant clinical benefit [17], while transgenic *mdx* mouse studies by Wells *et al* 1995 [18], showed that mini-dystrophin levels of 20-30%, relative to wild type, reduced dystrophic pathology.

Dystrophin detected by immunostaining on tissue sections or by western blotting can be tedious, technically challenging, and difficult to standardize. Both approaches require tissue obtained by muscle biopsy, an invasive and costly procedure for all patients. Repeated muscle biopsy of DMD patients, nearly all of whom are children, is not an option and alternative dystrophin analysis techniques are urgently required. Full-length muscle dystrophin, expressed in melanocytes, [19] could potentially be used to monitor dystrophin expression and function, but will need to be further evaluated. An additional limitation of dystrophin detection in muscle biopsies is that the sample may not reflect the expression pattern in other muscles, and furthermore, quantification of dystrophin expression on sections can be problematic. For all of these reasons, evaluation of modest changes in dystrophin expression after therapeutic intervention in dystrophic tissue presents a significant challenge.

With the advent of dystrophin restoring strategies and the need for meticulous evaluation of therapies, improvements in dystrophin detection and quantification have become an imperative, and two groups have published detailed methods for the unbiased quantification of dystrophin immunofluorescent expression [20, 21]. Detailed dystrophin quantification using one of these [21] along with clinical correlations in BMD patients clearly indicate that internally deleted dystrophin isoforms have the capacity to confer marked clinical benefits to individuals with DMD [22]. Anthony and colleagues [22] reported that muscle dystrophin

expression in BMD patients with a deletion end-point of exon 51 were higher than those in BMD patients whose deletions ended with exon 53. These results suggest that dystrophin expression and function will be influenced by the location and extent of the deletion, the abundance of the dystrophin isoform, disruptions to protein structure (such as spectrin repeats and crucial functional domains), and probably, additional, as-yet unidentified factors. Nevertheless, all varieties of internally deleted dystrophin that they studied were able to confer a clinical benefit, as shown by the BMD phenotype. This supports the hypothesis that low levels of dystrophin can confer substantial protection to the muscle, and the "threshold" level may be determined by the dystrophin quality.

Given the challenges of standardizing dystrophin quantification, other readily accessible biomarkers that reflect the presence or absence of dystrophin are being vigorously sought. Serum creatine kinase has long been known to be greatly elevated in DMD and BMD patients, but is also raised in other conditions, and it is highly variable. Serum creatine kinase levels do not reflect the extent of the pathology nor disease progression, and are subject to substantial variation due to stresses such as exercise. Several groups are evaluating changes in selected miRNA levels in serum. A distinctive serum miRNA profile was first identified in mice, leading to identification of dysregulated miRNAs as potential biomarkers in DMD [23]. Similarly, the serum levels of several miRNAs in *mdx* mice and the canine X-linked muscular dystrophy (Japan) model (CXMD(J)) were increased, but unlike creatine kinase levels, these miRNAs in *mdx* mouse serum were largely unaffected by exercise [24]. miRNAs abundant in the blood of *mdx* mice recovered to wild-type levels in mice effectively treated by AAV-mediated exon skipping [25], however, precise correlations between miRNA levels and disease stage, including extent of muscle degeneration, remain to be confirmed. Zaharieva and colleagues [26] reported a trend towards normalization of 'dystromirs' (circulating

miRNAs) in a subset of patients treated with eteplirsen and suggested that miR-1 and miR-133 could be considered as exploratory biomarkers for future studies.

Biomarkers providing predictive signatures of pathology, clinical status, or response to therapy are being used to develop diagnostic and monitoring tools that are rapid, economical, scalable, and versatile [27, 28] (for review see [29-31]). Circulating indicators of apoptosis, various cytokines [32], serum proteins in addition to serum creatine kinase [33], metabolites [34] and MRI techniques [35] are being investigated to monitor disease progression in muscular dystrophies, including DMD. However, the validity of such markers as surrogate indicators of dystrophin presence and functionality, and their utility in evaluating emerging therapies for DMD is unproven. Furthermore, the ability of surrogate biomarkers to discriminate therapy-mediated benefits from those due to variable (low) levels of endogenous dystrophin, frequently detected in DMD muscle [12, 36], remains questionable at this time. Studies in human subjects are clearly warranted and should be a priority of the DMD clinical research community, but until such time that surrogate biomarkers are validated, it is appropriate that dystrophin restoring therapies are assessed by the appearance of dystrophin.

Because of the well-documented detection of dystrophin in DMD muscle [12-14], it is reasonable to expect that the use of dystrophin as a treatment biomarker should require analysis of base-line expression. The sensitivity of current immunofluorescent quantification methods [20, 21] should allow reproducible fold-change quantification at even low levels of baseline expression, but whether dystrophin expression is measured by western blotting or by immunohistochemistry techniques, it is logical that fold changes and not fractional increases in dystrophin production above baseline will be needed to ameliorate disease progression in DMD. The minimally sufficient fold change necessary for functional improvement is

unlikely to be universally definable; it may differ for different therapies, as it may be dependent upon the induction of the specific dystrophin isoform resulting from therapy, the duration of treatment, and other factors. Nevertheless, the expectation of clinical improvement should follow that an increase in the expression of a dystrophin protein known to be associated with disease amelioration – as is the case with exon skipping therapies – is not only biologically plausible but is supported by the existing observational literature.

**References**

[1] Nicholson LV, Davison K, Johnson MA, et al. Dystrophin in skeletal muscle. II. Immunoreactivity in patients with Xp21 muscular dystrophy. Journal Neurol Sci 1989;94:137-46.

[2] Uchino M, Araki S, Miike T, Teramoto H, Nakamura T, Yasutake T. Localization and characterization of dystrophin in muscle biopsy specimens from Duchenne muscular dystrophy and various neuromuscular disorders. Muscle & nerve 1989;12:1009-16.

[3] Monaco AP, Bertelson CJ, Liechti-Gallati S, Moser H, Kunkel LM. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics 1988;2:90-5.

[4] Koenig M, Beggs AH, Moyer M, et al. The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. Am J Hum Genet 1989;45:498-506.

[5] Emery AE. The muscular dystrophies. Lancet 2002;359:687-95.

[6] Emery AE. Muscular dystrophy into the new millennium. Neuromuscul Disord 2002;12:343-9.

[7] Aartsma-Rus A, Janson AA, Kaman WE, et al. Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. Hum Mol Genet 2003;12:907-14.

[8] van Deutekom JC, Bremmer-Bout M, Janson AA, et al. Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. Hum Mol Genet 2001;10:1547-54.

[9] Wilton SD, Fall AM, Harding PL, McClorey G, Coleman C, Fletcher S. Antisense Oligonucleotide-induced Exon Skipping Across the Human Dystrophin Gene Transcript. Mol Ther 2007;15:1288-96.

[10] Fanin M, Freda MP, Vitiello L, Danieli GA, Pegoraro E, Angelini C. Duchenne phenotype with in-frame deletion removing major portion of dystrophin rod: threshold effect for deletion size? Muscle Nerve 1996;19:1154-60.

[11] Mendell JR, Rodino-Klapac LR, Sahenk Z, et al. Eteplirsen for the treatment of Duchenne muscular dystrophy. Annals of neurology 2013;74:637-47.

[12] Nicholson LV, Johnson MA, Bushby KM, Gardner-Medwin D. Functional significance of dystrophin positive fibres in Duchenne muscular dystrophy. Arch Dis Child 1993;68:632-6.

[13] Gangopadhyay SB, Sherratt TG, Heckmatt JZ, et al. Dystrophin in frameshift deletion patients with Becker muscular dystrophy. Am J Hum Genet 1992;51:562-70.

[14] Hodgson S, Hart K, Abbs S, et al. Correlation of clinical and deletion data in Duchenne and Becker muscular dystrophy. Journal Med Genet 1989;26:682-93.

[15] Chelly J, Gilgenkrantz H, Hugnot JP, et al. Illegitimate transcription. Application to the analysis of truncated transcripts of the dystrophin gene in nonmuscle cultured cells from Duchenne and Becker patients. Journal Clinical Invest 1991;88:1161-6.

[16] Chelly J, Gilgenkrantz H, Lambert M, et al. Effect of dystrophin gene deletions on mRNA levels and processing in Duchenne and Becker muscular dystrophies. Cell 1990;63:1239-48.

[17] Sharp PS, Bye AJH, Wells DJ. Physiological Characterization of Muscle Strength With Variable Levels of Dystrophin Restoration in *mdx* Mice Following Local Antisense Therapy. Mol Ther 2010.

[18] Wells DJ, Wells KE, Asante EA, et al. Expression of human full-length and minidystrophin in transgenic *mdx* mice: implications for gene therapy of Duchenne muscular dystrophy. Hum Mol Genet 1995;4:1245-50.

[19] Pellegrini C, Zulian A, Gualandi F, et al. Melanocytes--a novel tool to study mitochondrial dysfunction in Duchenne muscular dystrophy. Journal Cell Physiol 2013;228:1323-31.

[20] Taylor LE, Kaminoh YJ, Rodesch CK, Flanigan KM. Quantification of dystrophin immunofluorescence in dystrophinopathy muscle specimens. Neuropath Applied neurobiology 2012;38:591-601.

[21] Arechavala-Gomeza V, Kinali M, Feng L, et al. Immunohistological intensity measurements as a tool to assess sarcolemma-associated protein expression. Neuropathol Appl Neurobiol 2010;36:265-74.

[22] Anthony K, Cirak S, Torelli S, et al. Dystrophin quantification and clinical correlations in Becker muscular dystrophy: implications for clinical trials. Brain 2011;134:3547-59.

[23] Vignier N, Amor F, Fogel P, et al. Distinctive serum miRNA profile in mouse models of striated muscular pathologies. PLoS One 2013;8:e55281.

[24] Mizuno H, Nakamura A, Aoki Y, et al. Identification of muscle-specific microRNAs in serum of muscular dystrophy animal models: promising novel blood-based markers for muscular dystrophy. PLoS One 2011;6:e18388.

[25] Cacchiarelli D, Legnini I, Martone J, et al. miRNAs as serum biomarkers for Duchenne muscular dystrophy. EMBO Mol Med 2011;3:258-65.

[26] Zaharieva IT, Calissano M, Scoto M, et al. Dystromirs as serum biomarkers for monitoring the disease severity in Duchenne muscular Dystrophy. PLoS One 2013;8:e80263.

[27] Mehan MR, Ostroff R, Wilcox SK, et al. Highly multiplexed proteomic platform for biomarker discovery, diagnostics, and therapeutics. Adv Exp Med Biol 2013;735:283-300.

[28] Wang C, Fang X, Lee CS. Recent advances in capillary electrophoresis-based proteomic techniques for biomarker discovery. Method Mol Biol 2013;984:1-12.

[29] McDermott JE, Wang J, Mitchell H, et al. Challenges in Biomarker Discovery: Combining Expert Insights with Statistical Analysis of Complex Omics Data. Expert Opin Med Diagn 2013;7:37-51.

[30] Monteiro MS, Carvalho M, Bastos ML, Guedes de Pinho P. Metabolomics analysis for biomarker discovery: advances and challenges. Curr Med Chem 2013;20:257-71.

[31] Wang IM, Stone DJ, Nickle D, Loboda A, Puig O, Roberts C. Systems biology approach for new target and biomarker identification. Curr Top Microbiol Immunol 2013;363:169-99.

[32] Abdel-Salam E, Abdel-Meguid I, Korraa SS. Markers of degeneration and regeneration in Duchenne muscular dystrophy. Acta myologica : myopathies and cardiomyopathies 2009;28:94-100.

[33] Nadarajah VD, van Putten M, Chaouch A, et al. Serum matrix metalloproteinase-9 (MMP-9) as a biomarker for monitoring disease progression in Duchenne muscular dystrophy (DMD). Neuromuscul Disord 2011;21:569-78.

[34] Martins-Bach AB, Bloise AC, Vainzof M, Rahnamaye Rabbani S. Metabolic profile of dystrophic mdx mouse muscles analyzed with in vitro magnetic resonance spectroscopy (MRS). Magn Reson Imaging 2012;30:1167-76.

[35] Hsieh TJ, Jaw TS, Chuang HY, Jong YJ, Liu GC, Li CW. Muscle metabolism in Duchenne muscular dystrophy assessed by in vivo proton magnetic resonance spectroscopy. J Comput Assist Tomogr 2009;33:150-4.

[36] Fletcher S, Adkin CF, Meloni P, et al. Targeted exon skipping to address "leaky" mutations in the dystrophin gene. Mol Ther Nucleic Acids 2012;1:e48.