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Personal Comment

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 (e.g. 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

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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 min 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 20 years ago, low-level dystrophin expression in DMD patient muscle was reported [12–14], and Nicholson et al. 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. [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 et al. [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