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COMMENTARY

What Can We Learn From Clinical Trials of Exon Skipping for DMD?

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On 20 September 2013, GlaxoSmithKline (GSK) and Prosensa announced that GSK's Phase III clinical trial (NCT01254019) of Drisapersen, an exon skipping drug for Duchenne muscular dystrophy (DMD), failed to meet the primary endpoint of a statistically significant improvement in the 6 Minute Walking Distance Test (6MWT) compared to placebo.[1] On 12 November 2013, Sarepta Therapeutics announced that the US Food and Drug Administration (FDA) has considered its application for accelerated approval of Eteplirsen as a DMD drug to be premature.[2] The news came as a great disappointment to the scientific community and more specifically to the families and foundations that had followed the trail of research articles and press announcements. Moreover, the clinical trial results and the FDA's view of the relationship between the dystrophin biomarker and functional endpoint of 6MWT in clinic will have a far reaching impact beyond exon skipping therapy in DMD.

Currently, the most promising therapies for DMD are arguably gene therapy and exon skipping, both aiming to restore the expression of dystrophin. For exon skipping, the general strategy of restoring expression of the mutated dystrophin gene by excluding frame-disrupting mutations was vindicated by early experiments in dystrophic animal models.[3–6] This principle has been substantiated for DMD by clinical trials over the last 7 years with two chemistries, the 2'O-methyl phosphorothioate backbone (2OMePS, named PRO051/Drisapersen initiated by Prosensa/GSK) and the morpholino backbone (PMO, named Eteplirsen initiated by AVI Biopharma, now Sarepta Therapeutics).[7–10] Both drugs target dystrophin exon 51 and both elicited the expected skipping of exon 51 and production of dystrophin protein following intramuscular injections.

In a subsequent open-label, dose-escalation systemic study, five weekly subcutaneous injections of Prosensa/GSK's PRO051 at 0.5, 2, 4, or 6 mg/kg induced skipping of exon 51 accompanied by low levels of dystrophin in 12 DMD boys. But, importantly, this data lacked pretreatment controls.[10] This lack of pretreatment controls in combination with the use of the highly sensitive dystrophin antibody MANDYS106 led to reports of expansion of dystrophin positive fibre counts to up to 100%. The follow-up extension for 12-weeks at 6 mg/kg of the PRO051 reported stabilisation of motor function in the boys.[10] However, the study included several boys below 7 years of age where natural history would predict improved motor function within the experimental period. A subsequent Phase IIb placebo-controlled 6 mg/kg/week study (NCT01153932) of Drisapersen on 53 DMD patients again reported a significant benefit in 6MWT in the treatment over the placebo group. Clinical benefits were maintained, but with reduced significance, after 48 weeks of treatment. However, no muscle biopsy results have yet been reported.[11] This was followed by the phase III trial, with 186 patients, that failed to show statistically significant improvements in the primary outcome measure of the 6MWT. The difference in conclusion between the Phase III and earlier studies with the exact same treatment regime is therefore attributed almost solely to the high variability of the 6MWT endpoint measurement within the time-frame of the subject population and difference in sample sizes.

What have we learnt from the Prosensa/GSK trials? According to the comment from the FDA in response to Sarepta's application for Eteplirsen targeting the same dystrophin exon 51, the failure of the Drisapersen trial indicates a "*disconnect between increased expression of dystrophin and clinical efficacy*." This criticism has serious implications far beyond exon skipping in DMD raising questions about our assessment of all experimental therapies that aim to restore or produce functional dystrophins in DMD, including gene replacement therapies that deliver a known functional gene product as a drug.

So, is there clear evidence indicating a disconnect between the levels of dystrophin expression and clinical efficacy? Fortunately, the answer is no. Results, both from animal models[6] and clinical studies on Becker muscular dystrophy (BMD) patients, all point to a positive connection. Much of the confusion about levels of dystrophin expression come from over emphasizing assessment of dystrophin expression solely by immunohistochemistry (IHC) without pretreatment control biopsies for each patient and the use of highly sensitive antibody MANDYS106. Prosensa reported that up to 100% of fibers were dystrophin positive in the muscle samples of some Drisapersen-treated patients in its phase II study.[10] IHC is critical to provide us with a rough estimate of the levels of protein expression, the proportion of cells expressing the protein, and especially patterns of distribution. However, one must be aware that judgment of positivity of dystrophin

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staining with IHC is highly subjective and especially troublesome in DMD samples due to many factors, including the degeneration-related background staining, and highly variable distribution of both revertant fibers (spontaneously expressing dystrophin) and antisense oligomer-induced dystrophin expression, all of which call for comparison with a pretreatment biopsy. Small areas of clustered revertant fibers, reaching a few dozen or more, could easily be misinterpreted to result from treatment. For these reasons, IHC, even though very valuable for assessing distribution and localization, should never be the sole source of evidence for levels of dystrophin expression and require confirmatory backup.

Currently, the most generally available and reliable assessment of dystrophin levels in muscles is by western blots although the data are still, arguably semiquantitative, and it is generally difficult to convincingly demonstrate levels of dystrophin below 10% of normal levels. However, dystrophin levels higher than 10% of normal level can be demonstrated without much difficulty in most laboratories as observed from western blots using tenfold dilutions of positive control protein.[7,8,10] Unfortunately, data from western blots in the Drisapersen systemic trials showed only trace amounts of dystrophin in the treated muscle biopsy samples.[10] In fact, the image presented in the publication shows no clear difference in signal intensity between the baseline samples and those from 2/7 weeks after treatment.[10] The FDA also cited the failure of PTC Therapeutics's Ataluren trial as another example of the disconnect between levels of dystrophin and clinic outcomes. Ataluren has been selected to induce significant read-through of nonsense mutations of DMD gene, resulting in restoration of dystrophin expression in patients carrying such mutations. However, the screening assay method initially used to identify the specificity of PTC124 has been contradicted by more recent study.[12] Furthermore, studies to validate the effect of PTC124 in vitro did not demonstrate any significant read-through of nonsense mutations.[13] More importantly, clinical trials of Ataluren have shown only the phase IIa results with little evidence of dystrophin restoration by IHC with a very subjective scoring method.[14] Unfortunately, the phase IIb muscle biopsy results have not been presented and reported to the public. Therefore, the disappointing functional data from the Ataluren and Drisapersen trials are, in fact, entirely consistent with the failure of either agent to induce production of significant amounts of dystrophin protein and do not support the notion of the "disconnect" proclaimed in the FDA report. The rationale of both exon-skipping and stop-codon read-through is that any benefit derives from production of significant amounts of dystrophin and the degree of efficacy should be related to the level of dystrophin. This has been clearly demonstrated in animal models of DMD.[6,15] This conclusion is also consistent with the data from Sarepta's clinical trials of the exon skipping drug Eteplirsen (formerly AVI-4658). The first open label, dose escalation (cohorts 1–6: 0·5, 1, 2, 4, 10, and 20 mg/kg bodyweight respectively), repeated intravenous administration study showed that Eteplirsen was well tolerated. There was a statistically significant dose-response as well as remarkable variability in dystrophin production in patient muscles. In the low dose cohorts 1–4, there was no increase in dystrophin expression, with the exception of one subject in cohort 3. However, 6 of 8 subjects in the two highest dose cohorts 5 and 6 showed an increase in dystrophin expression. Three patients, one in each of cohorts 3, 5, and 6, showed a substantial number of dystrophin-positive fibers, 21, 15, and 55%, respectively. Western blot analysis of these patients also showed an increase following treatment of protein levels from 2 to 18%, from 0.9 to 17% and from 0 to 7.7% of normal muscle values, respectively.[8] This was followed by a placebo-controlled phase IIb trial using higher doses (30 and 50 mg/kg/week, each cohort had $n = 4$ subjects) of Eteplirsen.[16] Ambulant boys were treated for 24 weeks and primary outcome was the percentage of increase in the number of dystrophin positive fibres in comparison to baseline biopsies. The secondary outcome was safety and the 6MWT. After another 24 weeks of open label extension, a final muscle biopsy was taken at 48 weeks. The results showed the number of dystrophin positive fibres up to 52 and 43% in the 30 and 50 mg/kg cohorts respectively.[16] Overall, the Eteplirsen trials showed a clear trend of dose-dependent increase in dystrophin expression.

Clearly, one major difference between the two chemistries of exon skipping clinic trials is that Eteplirsen has been administrated systemically at up to 50 mg/kg, more than eight times higher than Drisapersen. The limited dose of 6 mg/kg for Drisapersen was largely the result of kidney toxicity whereas no clear toxicity has so far been identified for Eteplirsen. Most preclinical studies have reported higher efficiency of exon skipping with the PMO chemistry than with equivalent amounts of reagent based on the 2OMePS chemistry.[4–6] It is therefore not surprising that the Eteplirsen trial reported detectable dystrophin by western blot. The Eteplirsen treated patients also showed a benefit of 67 m less decline in 6MWT over the placebo group.[16] Stabilization of the motor function in DMD boys participating in Eteplirsen extension study has now being observed over 2 years. However one needs to be aware of the small sample sizes (only 12 subjects) and the bias associated with the open label nature of the extension study. Nevertheless, the levels of dystrophin were limited, clearly below 10% by western blot, and from only one sample (judged from the image presented in the publication) despite the high percentage of dystrophin positive fibers reported.[16] This again illustrates the inflation of using IHC and number of dystrophin positive fibers as a marker of overall levels of dystrophin expression. One also needs to be aware that similar results of 6MWT stabilization have been reported from the Drisapersen open label extension studies and recently published natural history studies, although the interpretation is complex because of different age ranges and subpopulations.[17,18] It therefore remains to be seen whether the reported levels of dystrophin under the current Eteplirsen treatment regime can be maintained. Upcoming placebo controlled phase III studies will be able to show if Eteplirsen can significantly delay the long-term disease progression.

There should be no doubt that dystrophin is critical for normal muscle functions, for its lack is the cause of DMD and BMD.[19] One could argue that increase of any amount of dystrophin might benefit the dystrophic muscle. However, the presence of low amounts of dystrophin in these trials is associated with limited distribution of the dystrophin positive fibers. The small number of these fibers in dystrophic

muscles is unlikely to provide widespread and measurable functional improvement to the general mass of muscle, a view supported by studies in animal models.[4–6] Therefore, therapy based on dystrophin restoration must ask the question: can the treatment produce sufficient amounts of dystrophin, measureable by both IHC and western blot, and sufficiently widely distributed, to effectively delay the disease progression? To answer this question, one would first ask how much dystrophin is required to have a significant impact on the disease outcome. Analyses of biopsies from BMD patients provide some insights. BMD is associated with natural occurring in-frame deletions from which reduced amounts of slightly shortened dystrophins are produced. The aim of exon skipping therapy in DMD patients is to mimic BMD by causing production of in-frame transcripts from the out-of-frame dystrophin gene. Published work on dystophin levels in near-asymptomatic Becker patients suggests that this degree of amelioration can result from around 30% of normal dystrophin levels in western blots.[20] Dystrophinopathy patients of intermediate clinical severity have been associated with dystrophin levels of between 10 and 25% of normal levels[21] while in-frame deletions in BMD patients with severe DMD phenotype have been associated with less than 10% dystrophin. From this data, it is reasonable to postulate that significant improvement in the disease phenotype by exon skipping will require expression of truncated dystrophin to at least 10% of normal levels with wide distribution. Such levels of dystrophin have not so far been convincingly demonstrated in any of the systemic trials of exon skipping and read-through treatments. Significantly higher levels of dystrophin are likely required to improve disease phenotype of individuals with diminished remaining muscle mass and higher variation in dystrophin distribution. This assumption does not exclude possible limited benefit to diseased muscle of lower levels of dystrophin, but the incidence of severe BMD patients with low levels of dystrophin indicates that this is unlikely.

With all the data available from animal model studies of exon skipping and adeno-associated virus gene therapy and clinic trials of exon skipping, we may conclude that dystrophin protein level, far from being dubious, is a fundamental biomarker to be taken into account when assessing experimental therapies that aim to produce and restore the expression of dystrophin in DMD. This principle is applicable to any therapy that aims to produce therapeutic protein.

We are still in the early stages of clinical trials for the exon skipping therapy. It is therefore critical to collect such data as accurately as possible, so that real guidance may be provided for later clinical trials targeting other exons or for gene replacement therapy. The efforts made by several large collaborative groups of physicians and scientists in the design and execution of these large clinical trials can't be appreciated enough as we have learnt invaluable lessons about the natural history of DMD, the practicability and utility of clinical trial procedures and outcome measures, and have created a sustainable clinical infrastructure for future clinical trials. The most important lesson learnt is that, clinical endpoints such as 6MWT are important for assessing the disease progression, but total dominance of this principle appears to have led to the misconception that clinical benefit can be achieved without the production of a therapeutic amount of relevant protein product.

Another valuable and important lesson from the two exon skipping trials is that the behaviour of a particular antisense chemistry in the mouse and dog models appears to provide a good guide to what it will do in human DMD. The relationship of dose per body weight to exon-skipping efficiency and dystrophin production with the PMO chemistry in animal models has been strongly paralleled in man. It reinforces the rationale for conducting initial investigations of the efficacy/toxicity window for a given chemistry in animals with a reasonable expectation that the general principles of dose and regime may be transferable to man.

In summary, detection of dystrophin is fundamental to exon-skipping therapy in DMD and it is important to gather reliable data relating levels of dystrophin expression to clinic outcome. This will provide guidance for later trials testing other antisense chemistries and targeting other exons as well as for gene replacement therapy. Preclinical studies in the dystrophic mouse and dog have shown a dose dependent dystrophin induction and therapeutic effect on dystrophic muscles. In fact, clinical and biochemical outcome from the treatment regimes of the two different chemistries are strongly predicted from animal model studies. Data from the pivotal Ataluren and Drisapersen clinical trials of exon skipping have, so far demonstrated to our best knowledge too little dystrophin to justify the notion of a disconnection between the levels of dystrophin expression and clinical outcome measurement. The newly started Ataluren phase III trial (NCT01826487) might be able to determine clinical benefit to DMD patients, but unfortunately no muscle biopsies have been planned in this phase III study. Determination of dystrophin levels should rely more heavily on western blot, currently the most available method, with supporting data from IHC, although more reliable and accurate detection methods are currently being developed.[22] Lastly, we would remind the FDA and ourselves that dystrophin is not a purely phenomenological biomarker for DMD; lack of dystrophin is the primary biochemical defect in DMD and denial of the importance of the principle of substantial dystrophin restoration in DMD would have future impact on all therapeutic investigations of inherited disorders.

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