Strapline: Neuromuscular disease

Why dystrophin quantification is key in the eteplirsen saga

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Eteplirsen, a compound designed to restore dystrophin in patients with Duchenne muscular dystrophy, controversially received approval by the FDA in 2016. Owing to limited clinical data, the approval was based on eteplirsen's effect on dystrophin expression. Now, the dystrophin quantification results have been published and although low levels of dystrophin expression are shown, the quantification remains debatable.

Refers to Charleston, J. S. et al. Eteplirsen treatment for Duchenne muscular dystrophy: Exon skipping and dystrophin production. *Neurology*, https://dx.doi.org/10.1212/WNL.0000000000005680 (2018).

Duchenne muscular dystrophy (DMD) is a fatal, X-linked progressive muscle-wasting disease caused by mutations (generally large deletions) that abolish production of dystrophin. This protein links the cytoskeleton to the extracellular matrix of muscle fibers and so protects them from being damaged during contraction. Deletions that are located in the middle of the gene and maintain the reading frame allow the production of short, partially functional dystrophin proteins. These 'pseudodystrophins' are associated with Becker muscular dystrophy (BMD), a muscular dystrophy that has a later onset and a slower disease progression than DMD, with near-normal life expectancy.

Eteplirsen is a so-called exon-skipping drug. The exon-skipping approach aims to manipulate the splicing of dystrophin transcripts in patients with DMD such that the reading frame is restored, enabling these patients to produce BMD-like pseudodystrophins. The approach is mutation specific, as the size and location of the deletion dictates the exon to be targeted by the therapy. Eteplirsen's therapeutic mechanism of exon 51 skipping is applicable to ~14% of patients with DMD.

Eteplirsen was approved under the accelerated approval pathway, which facilitates the approval of agents for serious or life-threatening diseases, enabling patient access to new therapies while the company conducts clinical trials to verify the predicted clinical benefit. The approval was based on a study done in 12 patients without a placebo control. Although the sponsor (Sarepta Therapeutics) argued that eteplirsen treatment resulted in a reduced disease progression in treated patients compared with natural history data, the FDA did not accept this finding as proof of any functional effects. However, FDA granted accelerated approval on the basis of the surrogate endpoint of dystrophin increase in skeletal muscle and Sarepta was requested to provide data confirming functional effects by 2021 as part of the conditions of the accelerated approval[1].

In a new paper, published 18 months after the approval, Charleston et al.[2] report the quantification of dystrophin in the biopsies collected in the eteplirsen trial. Patients were first treated for 24 weeks with placebo or eteplirsen (30 mg/kg or 50 mg/kg) via weekly intravenous infusion. However, after 24 weeks the four patients who received placebo were included in the treated cohorts and an open label trial was run for over 3 years. Muscle biopsies were obtained from all patients before treatment, after 12 weeks (50 mg/kg dose and two placebo treated patients), 24 weeks (30 mg/kg dose and two placebo treated patients) and 48 weeks (all patients). During the discussions with the FDA, a fourth biopsy was collected after 180 weeks of treatment from 11 of 12 patients.

Analysis of RNA extracted from these biopsies by reverse transcription PCR (RT-PCR) confirmed exon 51 skipping at the RNA level in all biopsies. However, RT-PCR can only confirm eteplirsen's mechanism of action, which might not be accompanied by dystrophin expression. To confirm dystrophin expression, several methods were used (FIG. 1): western blotting and two immunofluorescence analysis methods, one in which dystrophin-positive fibers were counted subjectively in a blinded fashion in samples from treated patients and another that used an automated digital image analysis system. Immunohistochemical analysis of muscle sections revealed dystrophin-positive fibres in muscle from patients treated for at least 24 weeks. Following suggestions from the FDA, a quantitative western blot was set up[3]. Notably, this undertaking is no mean feat, as dystrophin is a notoriously difficult protein to quantify, owing to its low abundance and high molecular weight[4].

Unfortunately, dystrophin quantification, particularly by western blotting, requires large amounts of sample and sufficient residual sample was available from the pretreatment biopsies of only three patients, so the investigators decided to use muscle biopsies from nine other patients with deletions treatable with eteplirsen as baseline samples. Western blot analysis showed that the levels of dystrophin in untreated patients varied between undetectable and 0.37%, whereas the levels after treatment varied between undetectable and 2.47%. Dystrophin was undetectable in the samples from six of nine untreated patients, whereas it was undetectable in the samples from only two of 11 treated patients. On average dystrophin expression was 0.93% in samples from treated individuals and 0.08% in untreated individuals. Immunohistochemical analysis of biopsies showed that the average fluorescence intensity in arbitrary units was 9.41 for the untreated patients and 22. for the treated patients, whereas the mean number of positive fibres was 1.12% and 17.39% for untreated and treated patients, respectively.

Consequently, it is very likely that eteplirsen induced an increase in dystrophin expression; however, we do not consider it to be possible to accurately quantify this increment with the data provided. The authors claim an 11.6-fold increase in dystrophin levels, which sounds very impressive. We would argue, however, that this claim is unsubstantiated. Most patients with DMD produce some dystrophin and these levels vary between patients[5]. As such, it is not possible to determine the fold-increases in the levels of dystrophin when baseline levels are not available (which was true for the majority of patients in this study) or below the lower limit of quantification (which was true all patients from whom pretreatment biopsy material was available). Furthermore, the lower limit of quantification of the assay is 0.25% dystrophin[3], but the authors nevertheless used levels below this value detected in unrelated patients to quantify fold increases. Only three of the patients had pre-treatment and post-treatment biopsies that could provide acceptable data: these individuals all show an increase of dystrophin expression by

immunohistochemistry (from 7.4-16.3 to 26.7-32.5 arbitrary units), which corresponds to a very small amount of dystrophin.

The clinical relevance of this increase is debatable and this issue probably underlies the reticence of the European Medicines agency (EMA) to follow the FDA's lead and approve eteplirsen in Europe. There are arguments for and against the benefit of very small levels of dystrophin. Within the past few years, it has become clear that patients who produce low levels of dystrophin from birth have a slower disease progression than those who produce dystrophin levels below the detection limit, and in a mouse model of severe DMD, dystrophin levels below 4% were sufficient to increase median survival from ~3 months to ~7 months[6]. However, these patients and mice produced these dystrophin levels from birth. Whether restoration of dystrophin in patients with DMD at these levels but at a later time-point will result also in a slower disease progression remains to be confirmed.

Several confirmatory studies to test functional effects of eteplirsen treatment are ongoing and will hopefully solve questions about eteplirsen's clinical use. If these clinical results are still unclear, validated and stringent dystrophin quantification methods might facilitate the evaluation of these agents, but will require of the use of pre-treatment and post-treatment biopsies.

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