Report of a TREAT-NMD / World Duchenne Organisation meeting on dystrophin quantification methodology

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Abstract

Representatives of academia, patient organisations, industry and the United States Food and Drug Administration attended a workshop on dystrophin quantification methodology. The aims of the workshop were to provide an overview of methods used to quantify dystrophin levels in human skeletal muscle and their applicability to clinical trial samples, outline the gaps with regards to validating the methods for robust clinical applications prior to regulatory agency review, and to align future efforts towards further optimizing these methods. The workshop facilitated a constructive but also critical discussion on the potential and limitations of techniques currently used in the field of translational research (western blot and immunofluorescence analysis) and emerging techniques (mass spectrometry and capillary western immunoassay). Notably, all participants reported variation in dystrophin levels between muscle biopsies from different healthy individuals and agreed on the need for a common reference sample.

Introduction

Thirty one representatives of academia, patient organisations, industry and the United States Food and Drug Administration from 6 countries (USA, UK, the Netherlands, Spain, France and Switzerland) attended the TREAT-NMD and World Duchenne Organisation workshop on dystrophin quantification methodology organized by Annemieke Aartsma-Rus (Leiden University Medical Center, the Netherlands and John Walton Muscular Dystrophy Research Center, Newcastle University, UK), Virginia Arechavala-Gomeza (Biocruces Health Research Institute, Spain) and Francesco Muntoni (University College London, UK), and sponsored by Duchenne Parent Project (the Netherlands). The meeting was held in London on the 14th March 2018.

Dystrophin quantification in context

Francesco Muntoni outlined the aims of the meeting and the current relevance of dystrophin quantification: multiple therapeutic approaches aim at dystrophin restoration for Duchenne muscular dystrophy (DMD), some of which have already been tested in trials, while many more trials are ongoing or being planned. Since the early days of these trials a decade ago, when it was realised that there was the need to measure with precision low levels of dystrophin following experimental therapies using antisense oligonucleotides (AON) and small molecules to induce read-through of nonsense mutations, the field has become increasingly aware of strengths and limitations of the methods used to measure dystrophin levels (1, 2).

The need for robust quantitation and regulatory compliance, with dystrophin measures as a primary outcome measure for clinical trials, has changed the way in which biochemical outcome measures are being assessed and considered (3): as an example, it was commonly believed that dystrophin was nearly absent from muscles from DMD patients, aside from rare revertant fibers (dystrophin positive fibres seen in many DMD patients) (4). It is now clear that most patients also produce trace amounts of

dystrophin in non-revertant fibers (4, 5). Therefore, pre-treatment biopsies are needed to accurately quantify dystrophin produced by a given treatment. It has also been demonstrated that while individual patients may have variable levels of low / trace amounts of dystrophin, there are clear trends with some genotypes that are associated with overall higher levels of dystrophin expression than others. For example, patients harbouring deletions flanking exon 44 produce higher amounts of dystrophin and also have a slower disease progression compared to other genotypes (6, 7). Furthermore, different dystrophin restoration approaches aim to produce different kinds of proteins: while exon skipping and stop codon read through strategies would result in internally deleted but nearly full-length proteins, AAV mediated gene therapy trials aim at the expression of smaller engineered micro-dystrophins (8). The production of the latter is much easier to differentiate from baseline low levels of dystrophin in DMD patients, but the clinical relevance of *de novo* levels are also less clear, as these are not naturally occurring deleted proteins.

Current and future trials can benefit from what has been learned in the past, as well as from work that was inspired by the regulators requesting that dystrophin be quantified more objectively (1). A collaborative effort (9) in which western blot and immunofluorescence methods (10-12) were compared between different groups with expertise in dystrophin quantification, revealed that only after a careful standardisation of the protocols was completed were the outputs comparable (9). In some laboratories, the reproducibility and sensitivity of western blot appeared less satisfactory than digital immunohistochemical capture, especially when measuring the low levels of dystrophin that are seen in children treated with first generation drugs. However, for the immunohistochemical capture technique, the concern from regulatory authorities with regards to data reproducibility relates to potential bias in the selection of regions of interest in the biopsy, or on the subjective assignment of fibres as "positive" or "negative" based on visual examination alone, and challenges in normalizing to 100% dystrophin levels seen in normal control biopsies. Consequently, they recommend objective and automated

capturing of images so that consistent analysis can be performed by independent laboratories (https://www.fda.gov/downloads/advisorycommittees/committeesmeetingmaterials/drugs/peripheralandcentralnervoussystemdrugsadvisorycommittee/ucm497063.pdf) (13).

Regulators are increasingly requesting an 'orthogonal approach' – meaning simultaneous use of multiple methods in the same clinical trial biopsies, including western blotting, immunofluorescence, RT-PCR or others: western blotting can provide information on the size and relative amount of the protein extracted from the sample, while immunofluorescence reveals how dystrophin is distributed across the tissue sample and where it is located within the fibres.

Finally, since quantification is generally relative (percentage of normal), we need to define "normal". It is now known that dystrophin levels vary not only between different muscles of the same normal control, but also between different control individuals (10, 12, 14, 15). To allow comparison between different trials, there should ideally be agreement on a common standard (12). This is not straightforward, as it has not yet been possible to produce recombinant dystrophin protein that could be used as a protein standard (16).

Dystrophin protein

Annemieke Aartsma-Rus summarized our current knowledge on dystrophin protein. A frequent question is: how much dystrophin do we need? There is no simple answer to this question, because the level needed depends on other factors, such as the structure and functionality of the new protein, the amount that is produced, and the disease stage (that itself depends on age and genetic background of the patient) at which dystrophin is restored. The immunological and inflammatory processes associated with DMD and replacement therapy, as well as metabolic abnormalities responsible for the muscle pathology in DMD and ongoing regeneration, may well impact on the success of dystrophin-replacement strategies (17).

Which dystrophin

The kind of dystrophin protein produced varies according to the therapeutic approach. Normal muscle dystrophin is a 427 kDa protein that has 3 actin binding domains (encoded by exons 2-10 and 31-45), 24 spectrin-like repeats (encoded by exons 11-63), a cysteine rich domain involved in binding to beta-dystroglycan (encoded by exon 64-70) and a C-terminal domain (18). Stop codon read-through drugs used in patients with nonsense mutations could produce a full-length dystrophin with potentially one altered amino acid. Given the length of the protein, and the general tolerance for missense mutations in most regions except those directly involved with actin or dystroglycan binding (18), these dystrophins are theoretically anticipated to be more functional, although additional studies may be required to confirm functional activity in muscle fibers in DMD patient samples. Exon skipping in eligible patients with out-of-frame deletions will produce different dystrophins based on the size and location of the mutation to be targeted. Different "quasi-dystrophins" are likely to have different functionality, which could be partially anticipated based on the phenotype observed in Becker patients with the equivalent genomic deletions (19). However, not all dystrophins formed after exon skipping have an equivalent in Becker patients and it is known that mutations in several regions of dystrophin lead to more severe Becker phenotypes (e.g. mutations in the N-terminal end, which encodes the first two actin-binding domains), while others tend to be associated with much milder progression (e.g. mutations in the first part of the central repeat domain encoded by exons 11-30 (20)).

The spectrin-like repeats are generally observed as redundant parts of the protein. However, one should bear in mind that each individual repeat acts as a unit, and is connected to the next repeat in a head to tail manner. Exons generally do not encode full repeats, and after exon skipping this normal connection of one repeat to the next is often lost. In fact, there is a growing literature suggesting that the alignment of spectrin-like repeats in dystrophin after exon skipping influences functionality, probably more so in

heart than in skeletal muscles (21). For instance, in a dystrophin lacking the domains encoded by exons 45-55 (spectrin repeats 18-21 and parts of repeats 17 and 22), spectrin repeats 17 and 22 are joined almost seamlessly, while in a dystrophin lacking the domains encoded by exons 45-47, this is not the case (22). It is anticipated that binding of the dystrophin-associated proteins is not affected by the way the repeats are connected in an internally deleted dystrophin, since this is mediated by the domain encoded by exons 64-70. However, since histological analysis is done on skeletal muscle rather than heart, it cannot be excluded that the binding to dystrophin associated proteins may be affected in cardiac tissues (23).

Micro-dystrophins delivered by AAV are highly engineered proteins that contain only the most critical domains of the protein, i.e. the first 2 actin-binding domains, 4 spectrin-like repeats and the cysteine rich domain or other similar combinations (24, 25). Attempts have been made to obtain an optimal phasing organisation of the spectrin repeats. However, these dystrophins are a lot shorter than even the shortest Becker dystrophins that have been found to date. It has been confirmed that these dystrophins are functional in animal models (8) but not yet in humans. Also, it is not known which levels will be needed to achieve functional effects in humans (26).

How much dystrophin

Early studies of dystrophin protein correlations between western blot and clinical symptoms suggested that <3% normal levels typically showed a clinical picture of DMD, whereas >15% were more consistent with Becker muscular dystrophy (BMD) (27, 28). Intermediate levels (3-15%) were often seen with an intermediate phenotype (e.g. between DMD and BMD) and it was assumed that dystrophin restoring therapies should aspire to induce 20% to 30% of normal dystrophin levels (29, 30). More recently, Becker patients who produce lower than these originally suggested dystrophin levels (~10%) have been reported, and DMD patients with deletions flanking exon 44, who experience a slower disease

progression, also show low dystrophin levels , some below the level of detection by western blot (12, 15, 31). In mice lacking both dystrophin and utrophin, very low levels of dystrophin were sufficient to significantly improve survival: at 5 months all mice lacking dystrophin had perished while, 70% of mice with less than 5% of dystrophin were still alive (32). While it is not yet clear how this correlates to the human situation, it clearly shows that low amounts of dystrophin can have an impact on disease progression in mice. Dystrophin levels can also be modulated by inflammation and dystrophin-targeting microRNAs and long non-coding RNAs, adding more complexity to dystrophin protein and phenotype correlations (33).

The amount of dystrophin required to improve different aspects of muscle function is an important question that has been addressed in preclinical models. Exon skipping studies in *mdx* mice have shown that 5-15% of dystrophin is sufficient to protect muscle fibers against a force drop after tetanic contraction; however more than 40% dystrophin is needed to also improve muscle force (34). Similarly, in another study using a *mdx* mouse with low variable dystrophin levels, low amounts of dystrophin were able to improve muscle function but, to restore the neuromuscular junction defects, levels of 50% were needed (35). It is important to keep in mind the findings discussed earlier regarding dystrophin quantification variability when considering dystrophin levels reported in these studies.

In addition to the net levels of dystrophin produced, the distribution of dystrophin in muscle fibers is another factor that requires consideration. Analysis of biopsies collected in clinical trials of two different exon skipping antisense oligonucleotides (*2'-O*-methyl phosphorothioate (2OMePS) and morpholino phosphorodiamidate morpholino oligomer (PMO)) revealed differences in distribution possibly secondary to pharmacokinetic differences between the two chemistries: 2OMePS treatment resulted in widespread expression of dystrophin at very low levels (36), while PMO treatment resulted in higher dystrophin levels in only a smaller proportion of muscle fibres, in a patchy distribution (37, 38). It is not

yet known whether having either some dystrophin in all fibers, or higher dystrophin levels in some fibers, may provide a different therapeutic impact in the human. However, this quandary does emphasize the need for both western blot and immunofluorescence analysis; indeed a 2% dystrophin level detected by western blotting could reflect very different scenarios, for example, that all muscle fibers were producing 2% of dystrophin, or that 50% of muscle fibers were producing 4% of dystrophin or that 2% of fibers were producing 100% of dystrophin. It is likely that these different expression patterns will have different implications for function of the entire muscle.

Time of intervention

Most estimates of the amount of dystrophin needed for functional improvement are based on either BMD or X-linked dilated cardiomyopathy patients (30), DMD patients producing very low dystrophin amounts (6), or transgenic mouse models (32). However, in all of these, dystrophin has been produced from before birth, it is usually evenly distributed in muscle fibres, while when treating DMD patients, intervention takes place later. Restoring dystrophin later in life is not expected to bring back muscle tissue or function that has already been lost, and indeed the progressive muscle weakness that DMD patients experience is due to the substitution of muscle with fibroadipose tissue. As previously highlighted, the inflammatory environment of the DMD muscle fiber may also play a role in the impact of differential dystrophin restoration (33). As such, it is clear that treating earlier may lead to larger therapeutic effects, because the amount of remaining target tissue (muscle) would be larger and less function would have been lost. This was suggested in the double dystrophin/ utrophin knockout mice (39). Using a single treatment with a very high dose of an exon skipping antisense oligonucleotide, dystrophin levels were restored to almost 100% (i.e. almost 100% of muscle fibers produced close to 100% of dystrophin each). In young animals, this prevented pathology and increased survival to wild type levels. However, when treatment took place in older mice, 100% dystrophin restoration was

insufficient to improve muscle function, prevent kyphosis or, when given near the end of the lifespan, to improve survival. On the other hand, in a different set of experiments, when the same mice were treated from 1 week of age with oligonucleotides (200 mg/kg/week of 2OMePS), this resulted in very low levels of dystrophin restoration, which significantly improved survival (Aartsma-Rus et al., manuscript in preparation). Furthermore, it is known from a small study in Becker patients with the same deletion, that muscle strength and fatty infiltration as measured by MRI significantly correlated with patient age (7).

Patient perspective on muscle biopsies

Dystrophin analysis is performed on muscle biopsies. This is an invasive procedure in which care should be used to minimise pain and that is not without risk, since it is generally performed under general anaesthesia. Sejal Thakrar (Smile with Shiv, DMD patient organization, UK) presented a recent survey that was performed by the World Duchenne Organisation in collaboration with academic centres. The survey was answered by 78 parents and patients from 8 countries who had been involved in clinical trials that involved taking muscle biopsies generally from the tibialis anterior and biceps (Thakrar et al, manuscript in preparation). Parents report that informed consent documents do not always properly reflect the risks and processes of undergoing a muscle biopsy. Parents reported anaesthesia as the biggest perceived risk and while complications such as rhabdomyolysis are rare, the majority of patients (83%) suffered from pain after the biopsy that had a short-term impact on daily activities. In addition, most patients had a larger scar than they expected, or that was described in the information provided.

Parents find muscle biopsies acceptable in clinical trials, but in placebo-controlled trials many parents think the risk of having to undergo a muscle biopsy, while perhaps not receiving any drug, is very high. Parents also feel that remaining biopsy tissue should be shared with others for research purposes once the analyses related to the trial has been performed and that informed consent forms should include

this option. Recommendations from the patient community are that consent forms should inform patients accurately about not only the risks, but also the pain and the way this will be managed, the expected size of the scar and that surgery should ensure that the scar size is minimized and that parents and caregivers are informed of the results of the trials.

Regulatory perspective on dystrophin quantification

Ashutosh Rao (Food and Drug Administration (FDA), US) presented on analytical challenges and opportunities with dystrophin quantification methodology. Assay validation to confirm the suitability of the method and ensure reproducibility beyond exploratory findings can be challenging, but it is essential that this process is done prior to sample collection and analysis (40). The validation process is used to demonstrate that the analytical procedure employed for a specific test is suitable for the intended purpose, with regards to reproducibility, specificity, sensitivity, variability (intra-operator and inter-operator within the same lab, and also between different labs), accuracy, precision and range (especially at low levels) as acknowledged by consortia of experts such as the College of American Pathologists and detailed in publicly issued FDA guidance (41). For quantitative measurements, the validated ranges, including upper and lower limits of detection (LOD) and limits of quantitation (LOQ) can be important determinants of the limits of reliably reportable dystrophin levels with a particular method with its specific reagents (e.g. antibodies) and detection systems (e.g. chemiluminescence). With input from stakeholders, FDA's Center for Drug Evaluation and Research (CDER) has issued a guidance document on developing drugs for DMD (https://www.fda.gov/ucm/groups/fdagov-public/@fdagov-drugs-gen/documents/document/ucm450229.pdf). Dr Rao stressed that the FDA is aware that dystrophin quantification is challenging and is open to working with drug developers towards robust and objective methods for use in clinical studies. FDA encourages drug developers to engage with the agency early and often to allow a scientifically robust drug development pathway.

With regards to dystrophin quantification by western blotting, there are some specific challenges, starting with the variability between tissue lysis conditions, different electrophoresis and detection systems and the use of different dystrophin antibodies that preclude direct comparison between blots stained with different antibodies (42, 43). The large size and variants of dystrophin protein itself can also be challenging and often the methods were originally developed for the full-length dystrophin. Reliable quantification is generally conducted within the linear range of the reference sample concentration series, when the baseline sample, treated sample and standard are on the same blot and when there is no oversaturation (i.e. the intensities are within the linear dynamic range of detection). Comparison between studies is also complicated by the occurrence of double bands for the full-length protein, multiple bands for truncated versions (44, 45) and in some instances smears of closely-sized bands (42, 43). Some other areas that offer opportunity for improvement are the variation observed in baseline dystrophin levels within and between samples (11, 28), the need for a robust and reproducible dystrophin reference standard and having a good system to ensure sample integrity and blinding for multisite studies.

Immunofluorescence is a good method to confirm correct protein localization and allows automated and objective imaging with clinical trial samples. However, immunofluorescence is not a quantitative technique for protein levels, especially for a complex protein such as dystrophin (9). Dystrophin also presents unique challenges for immunofluorescence analysis due to the presence of high dystrophin levels in revertant fibres in DMD samples and the different levels of affinity and specificity of different anti-dystrophin antibodies (4, 10). Consensus is needed on how to objectively distinguish between dystrophin from revertant fibers and treatment-induced dystrophin. There is also a need to examine how the immunofluorescence results correlate with more quantitative methods such as western blot and establishing automated analyses with independent verification (40, 46). Reliance on a single analyst

and a single laboratory can result in problems of reproducibility when exploratory studies are expanded to support drug development.

There is room also for development of alternative methods to quantify dystrophin levels, especially at low levels. Mass spectrometry as an analytical tool for quantifying dystrophin levels has been published previously as a possible method, but thus far the accuracy and precision for levels of dystrophin below 5% have not been reported (47).

Immunofluorescence methods

Virginia Arechavala-Gomeza introduced an immunofluorescence method used for dystrophin quantification. Muscle transverse sections are stained with antibodies against dystrophin, which are then visualized by a fluorescently labelled secondary antibody. This approach can determine the number of dystrophin producing fibers as well as the amount dystrophin each fiber produces, although quantification is relative. Comparing samples from before and after treatment should reveal whether there is an increase in dystrophin production elicited by the treatment.

Several dystrophin quantification methods have been developed in parallel by different groups, with different degrees of operator dependency (10-12, 48). A comparison of the methods in DMD and Becker samples that were blindly provided to the different groups, established reasonable good comparability of the different methods once protocols were optimised (9). However, further optimization is needed for the very low ranges, i.e. the levels of dystrophin expected to be produced in a clinical trial.

Francesco Muntoni presented how the method developed by Dr Arechavala-Gomeza (10) has been adapted in an effort to make it operator independent, using Definiens software (48). Analysis revealed

that expression of laminin and spectrin was increased in DMD and Becker patients compared to controls. So while these proteins can be used as a 'mask' to identify the region of interest (i.e. where dystrophin is expressed) in an automated fashion, they should not be used for normalization as this may underestimate dystrophin expression. Using this automated method in the analysis of control samples it was observed that dystrophin levels varied in the same individual between samples from the same muscle and also from samples from different muscles (10). More normative data are needed for both patients and controls.

Rahul Phadke (University College London, UK) presented on a newly developed multiplex immune assay for multiparametric digital analysis. This method allows staining for multiple proteins using pre-adsorbed Alexa fluor conjugated secondaries and Thermo Fisher's Spectraviewer. This system allows also digital analysis in an unbiased and automated fashion for up to three different proteins, while staining of four or five proteins is being explored. Initial work indicates that Alexa 647 is brighter, more stable and less prone to bleaching.

Eric Hoffman ((Binghamton University-SUNY, and AGADA BioSciences) outlined processing of biopsies for a clinical trial of exon skipping, pointing out that duplicate biopsies and proper storage are crucial. AGADA BioSciences has produced a video on how they performed biopsy processing in the context of exon skipping clinical trials (https://www.youtube.com/watch?v=wyRoXgLDaF0). Also important are acceptance/rejection criteria: haematoxylin and eosin staining to confirm muscle quality and fiber orientation. Dystrophin immunostaining is done by double staining with dystrophin/laminin-α2 and dystrophin/alpha-sarcoglycan. Automated scans of complete slides are collected, and image analysis done on the stored images by two evaluators. Healthy muscle, non-treated DMD and Becker muscles are processed and analysed in parallel. The whole transverse section is analysed and the percentage of dystrophin-positive muscle fibers is calculated as well as the absolute intensity normalized to laminin-

α2 expression. A Becker sample expressing around 20% of dystrophin is used to establish the gain settings during the acquisition of the DMD samples.

Pallavi Lonkar (Wave Life Sciences) reiterated that, when analysing dystrophin restoration, the localization, percentage of positive fibers, the relative intensity of dystrophin and the distribution of dystrophin are important. She agreed that the entire cross section should be used for automated quantification and that post treatment samples should be compared to pre-treatment samples to assess whether there was a shift in dystrophin positive fibers and the relative amount of dystrophin produced.

During the discussion, it was highlighted that there is at the moment no single protocol used by all, which makes comparison between laboratories challenging. However, it is a good development that the field is moving towards an automated, operator independent method that analyses the whole cross section rather than selected fields.

When using dystrophin restoration as a surrogate endpoint, the presence of revertant fibers poses a technical challenge. The mechanisms through which these arise are not clear. It has been proposed that they are due to secondary, frame-restoring mutations in satellite cells or from epigenetic modifications in satellite cells that result in efficient frame-restoring exon skipping. Consequently, when satellite cells fuse with damaged fibers, or form new muscle fibers, these will become dystrophin positive (5). The vast majority of patients have revertant fibers, generally at less than 3% (4). In a small study it was shown that the number of revertant fibers within a patient over time is relatively stable (4). Trace dystrophin also appears to be stable over a period of 48 weeks in patients recruited in the placebo arm of a clinical trial (phase III, (49)).

There was discussion of using a reference protein for normalization. Given that the expression of many muscle membrane proteins are affected by the dystrophic pathology, normalizing dystrophin expression to a related reference protein may lead to over or underestimation. Care should therefore be taken in

ensuring that any reference protein has similar levels in control and dystrophic muscle. Dystrophin levels are sometimes given as relative amounts without normalization to a reference protein but this has its own challenges because the levels reported can be variable based on the specific method used , and in addition there is evidence for variability of the levels of production of these marker proteins between normal and dystrophic muscle (48).

Western blotting analysis

Annemieke Aartsma-Rus introduced western blotting as a technique where protein lysates are separated by size on acrylamide gels and then blotted on a membrane. After blocking, dystrophin is detected by dystrophin specific antibodies and visualized by secondary antibodies labelled with either electrochemoluminescencent (ECL) or infrared conjugates (Odyssey system from Li-Cor).

Because dystrophin is a large structural protein that can be difficult to fully solubilize, some labs use a combination of high detergent buffers (e.g. 10% SDS) and mechanical force (e.g. MagnaLyzer bead system). Because dystrophin protein is very sensitive to degradation, freeze-thaw cycles should be avoided and test and control samples should be aliquoted. Concentration series of the healthy control muscle extracts should cover the expected range of protein before and after treatment. In preclinical studies, the concentration series are often made in lysates from dystrophin negative *mdx* mouse muscle, to allow loading of equal total protein amounts. However, the availability of the human equivalent of a protein lysate from DMD patients with undetectable dystrophin levels is limited and as such, this approach is not easily used by all in a clinical trial setting.

Since dystrophin levels can vary between different muscles, it is recommended to use the same muscle types for samples and the concentration series. Furthermore, some have found as much as a six-fold difference between lowest and highest levels measured in healthy muscle (10, 12, 15). One can make a reference sample by mixing lysates from several individuals to reduce variation, or one can select a

sample that expresses a predefined, average amount of dystrophin and maintain a stock of these samples for the duration of studies.

Glenn Morris (Wolfson Centre for Inherited Neuromuscular Disease, UK) presented on the 154 dystrophin antibodies produced by his group since the early 90s (50) that have been deposited at the Developmental Studies Hybridoma Bank (Iowa University). Some antibodies detect fewer dystrophin degradation products than others (51), in particular carboxyl terminal antibodies. This has an impact on western blotting quantification when the amount of degradation varies between biopsies and quantification of the 427 kDa band alone would underestimate the amount of dystrophin present (if the biopsy sample shows dystrophin degradation). For mass spectrometry and immunofluorescence analysis, proteolysis may have a smaller impact on the interpretation, but antibodies containing a proteolytic cleavage site (in particular MANHINGE antibodies) should be avoided. Ideally one should of course avoid degradation, by flash freezing the biopsy sample immediately after surgery, and using well-characterized solubilization methods.

Kristy Brown (Solid Biosciences) emphasized the importance of optimizing all western blot steps and noted that selection of the primary antibody will greatly affect the optimization process. Under controlled conditions, western blotting is analytically robust. Analysis of micro-dystrophin protein by western blot has different analytical challenges than full-length dystrophin. She reiterated the variation observed in dystrophin protein levels in healthy muscle, where 30 normal human quadriceps samples expressed levels ranging from 60-130% of the average and suggested the possibility of using animal or control cultured cell lysates.

Eric Hoffman presented the western blotting methods used for an exon skipping clinical trial by AGADA BioSciences. Protein is isolated from cryo-sections in frozen Eppendorf tubes in a high SDS lysis buffer (10%SDS) at room temperature and then used immediately (no freeze/thaw cycles of solubilized

samples. Two normalizations were used: alpha-actinin immunoblot, and Coomassie Blue staining of the post-transfer gel. A standard curve was run on each blot, and was created using mixture of Normal (5 mixed samples all showing ~100% dystrophin), and a DMD patient (0% dystrophin) (5 point curve with 0, 1, 3, 10 and 25% of dystrophin). Each biopsy was analysed using three separate immunoblots (triplicate). Acceptance/rejection criteria were presented. Using this protocol, that uses ECL signal and a BioRad Chemidoc for data capture, the CV values are better than were reported in the past for low dystrophin levels.

Diane Frank (Sarepta Therapeutics Inc.) presented on the western blot protocol that is now run to assess samples from trials sponsored by Sarepta. Western blots are run under good laboratory practice (GLP) in a GCLP compliant laboratory and acceptance and quality control criteria are applied to each gel. Diane Frank reported how they deal with the variation in dystrophin levels in normal individuals by pooling samples from 11 non-BMD/DMD, histologically normal individuals. The dystrophin level of the pooled lysates approximated the level of the normal control previously used in clinical trial sample analyses. The pooled lysates have been aliquoted and are thawed no more than twice. A five point standard curve is used (0.25%, 0.5%, 1%, 2% and 4% of normal) and is diluted in DMD tissue to achieve equal protein load in each lane. ECL and films are used for detection. In a phase I/II study of SRP-4053 (golodirsen) in DMD patients (NCT02310906) 2 pre-treatment and 2 post-treatment lysates were analysed for each patient. The lower threshold of quantification for the method is 0.25%. Fifty gels were run successfully and all samples generated data, with a standard deviation of 13-49%. On average, there were 1.019% higher dystrophin levels in the post- compared to pre-treatment samples.

Pallavi Lonkar presented Wave Life Science's efforts to set up the western blot analysis. Because the reference curve is generally diluted in DMD muscle homogenate, setting up and running assays requires a lot of DMD tissue, and they have calculated that the analysis of a clinical trial would require 2 g of

DMD muscle or 200 muscle sections. In the absence of matched DMD tissue, alternate strategies were proposed to generate a standard curve by using either pooled DMD tissue lysate or *mdx* mouse pooled tissue lysate as a diluent. Performance of the assay was evaluated by accuracy of QC samples when calculated using the pooled DMD standard curve. Preliminary data suggested that pooled DMD homogenate or *mdx* pooled tissue lysate can be used as a suitable alternative diluent when preparing dystrophin standard curves for western blot. Pallavi Lonkar reported using α-tubulin as a reference protein rather than α-actinin.

During this session, the significant differences in protocols used became evident, with some laboratories using ECL and films, ECL and digitization, infrared labels and the Odyssey system from Li-Cor.

Emerging techniques

Eric Hoffman (AGADA BioPharma,USA) presented a mass spectrometry method that is in development, which can detect 1% dystrophin. This is an improvement over the previous version which had a lower limit of detection of 5%. Challenges are that peptide references are diluted in DMD muscle lysates, and this requires very large amounts of DMD muscle, and that the method requires expensive equipment for detection.

Hendrik Neubert (Pfizer) presented an immuno-affinity liquid chromatography (LC)-MS/MS detection system for dystrophin and micro-dystrophin quantification. The method is still in development and is designed to combine high measurement specificity with high sensitivity through enrichment with anti-peptide antibodies. Proteins are extracted from muscle samples with high (5%) SDS buffer followed by precipitation with an organic solvent, then proteins are re-solubilized and digested with trypsin. A multiplex peptide immuno-affinity extraction is performed, using antibodies against selected dystrophin

peptides prior to nanoflow/spray LC-MS akin to previously published methods for other protein analytes (52). Both fresh frozen samples and OCT embedded samples can be used. The protocol takes 1.5 days for sample preparation and 15 minute LC-MS run time per sample. Heavy isotope labelled micro-dystrophin or dystrophin peptides are used for response normalization. At the moment 4 peptides from different regions of the dystrophin protein have been prioritized, but additional peptides can be added if needed. The method currently uses an 11 point standard curve of recombinant micro-dystrophin, the lower limit of qualification is less than 1% of normal dystrophin, while the upper limit is 500%. The coefficient of variation (CV) is typically below 25%, even for the lower ranges of dystrophin levels. The method can measure dystrophin from various species (mouse, human) and detect dystrophin from revertant fibers in DMD patients at low levels (preliminary data were 1.6-5.9%). As before, dystrophin levels of healthy individuals varied.

Nicole Datson (BioMarin) presented a capillary western immunoassay (Wes) adapted to dystrophin (15). This method uses protein lysate samples in a plate, a capillary electrophoresis system and incubation with preloaded antibodies. The advantages are that the system is automated, that analysis is quick (3 hours), no gel running and blotting are required and only very low amounts of sample and antibody are used (100 fold less sample and 500 times less antibody than for a western bot). Four different antibodies which recognise different dystrophin epitopes were tested (NCL-dys1, Manx59b, Ab154168 and Mandys106) and all recognized the same peak at 300 kDa in a healthy control sample, which deviates from the predicted 427 kDa for the full-length (muscle) dystrophin isoform. Combined with the observation that this 300kDa peak had a smaller molecular weight in Becker samples and was absent in DMD samples, they concluded that this represents dystrophin and noted that the apparent molecular weight of large proteins above 280 kDa is often underestimated using Wes. This was corroborated by others who had used the system (Diane Frank and Silvia Torelli). Dystrophin quantification by Wes was highly sensitive and quantitative over a large dynamic range. Using 0.125 microgram of total protein,

levels from 0.5% (lower limit of quantification) to 100%, as well as minor increases in dystrophin levels (0.125%), were detectable. If needed, one can load 1.25 microgram protein. The reproducibility of dystrophin quantification by Wes is in line with the FDA/EMA regulations for quantitative biochemical analysis with a CV between replicate measurements generally below 20%, even in the lower range of dystrophin levels and between different operators. For normalization, alpha-actinin is used. However, since this protein is expressed at higher levels than dystrophin, samples need to be diluted and multiplexing is not possible.

As with other techniques, the dystrophin levels detected by Wes in skeletal muscle of 31 healthy controls varied by 3 to 5 fold, and the sample selected as a reference sample was one that had an average expression of the control samples.

Lower levels of dystrophin were found in samples from Becker patients (10-90%) and DMD patients (0.2-7%), the DMD patients with higher dystrophin levels having exon 44 skippable mutations.

Silvia Torelli (UCL) compared the Wes system to western blotting, using both ECL and infrared dyes as detection mechanisms. As controls, she used control muscle tissue spiked with 7 DMD samples. As reference proteins, she used either meta-vinculin (a muscle specific isoform of vinculin that is recognised by the vinculin antibody) or α-actinin. Using Wes, she was able to detect dystrophin in 0.0125 and 0.125 microgram of total protein and they report the same drawback described by Nicole Datson of not being able to use a loading control on the same capillary. In western blots, she favours wet transfer over the trans-blot system which gave inconsistent data, probably because this method is less sensitive. For signal capture, the Odyssey system was preferred to avoid saturation and benefit from Odyssey's operator-independent western analysis method. While the Mandys-106 antibody did not work on western blots, commercially available MABT827 works both on Wes and western blotting.

General discussion and alignment of future efforts

Patient representatives wondered whether historical controls could be used rather than pre-treatment samples. Given the variation observed between patients, this would only be possible when patients had frozen tissue available from a biopsy in the past for diagnostic purposes, which is now becoming a less common clinical practice. Furthermore, the fact that processing and storage influence the quality of the sample to a large extent, may render the use of historic controls challenging. Patient representatives further questioned whether biopsies are required in each dystrophin restoring trial. Once proof-of-mechanism is confirmed in an early phase trial using biopsies, they consider later phase trials as studies that should be focused on showing functional efficacy, although as some companies are focusing on early stage trials to opt for accelerated approval this may not be possible. The potential for needle biopsies was also mentioned.

It is clear that the amount of dystrophin protein expressed varies between healthy individuals. Therefore it is essential that there is agreement on what is considered 'normal' or used to set '100%' in relative measurements. Ideally a primary reference standard should be shared between the groups, allowing them to select a normal that expresses dystrophin at the same levels, or to all use the same reference. Then, different techniques (western blot, Wes and mass-spectrometry) should be compared using the reference sample to study variation in expression levels detected by the various techniques.

Another question was whether a reference protein needs to be included. For most protocols, the reference is used to confirm equal loading of pre- and post-treatment samples. However, when the muscle quality of pre- and post-treatment samples varies, one may also want to correct for muscle content. The proteins used most frequently appear to be α-actinin and meta-vinculin. The consensus appeared to be that using a reference protein is recommended as part of quality control, but that using it to correct is likely to introduce noise so should be used with caution.

Concluding remarks

In recent years, much progress has been achieved in the quantification of dystrophin. Our knowledge of the role of the protein has increased and techniques to reproducibly quantify small increases in dystrophin protein are now available (10, 12, 15, 48, 53). One drug has received accelerated approval in the US based on the surrogate endpoint of dystrophin increase (54) and there are currently confirmatory studies and other trials ongoing and planned aimed at dystrophin restoration, where the analysis is expected to be more robust due to the progress made. There are certain other insights that may facilitate future drug development. Aspects that require further work and joint effort include the following: First, now that we are aware that dystrophin levels vary between individuals, agreement is needed on a common reference sample, ideally kept in a biobank, such as the EUROBIOBANK. Second, academic groups and companies developing new dystrophin quantification techniques and/or working on dystrophin restoring therapies should initiate an early dialogue with the regulatory agencies. Finally, biopsies are invasive and should only be performed when this is crucial for a study, to confirm proof of mechanism for the test compound.

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Disclosures

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AAR discloses being employed by LUMC which has patents on exon skipping technology. As co-inventor of some of these patents AAR is entitled to a share of royalties. AAR further discloses being ad hoc consultant for PTC Therapeutics, BioMarin Pharmaceuticals Inc., WaVe, Sarepta Therapeutics, Global Guidepoint and GLG consultancy, Summit PLC, Grunenthal and BioClinica, being a member of the Duchenne Network Steering Committee (BioMarin) and of the scientific advisory boards of ProQR, MirXX Therapeutics and Philae Pharmaceuticals. Remuneration for these activities is paid to LUMC. LUMC also received speaker honoraria from PTC Therapeutics and BioMarin Pharmaceuticals.

H.N., J.O. and M.B. are employees of, and hold stock or stock options in Pfizer.

G.E. Morris reports no conflicts of interests.

P.L. is an employee of Wave Life Sciences.

EPH is co-founder and Vice President of AGADA BioSciences, co-founder and CEO of ReveraGen BioPharma, and co-founder and Board member of TRiNDS LLC. He owns stock and receives enumeration from each entity.

JD and ND are employees of, and hold stock or stock options in BioMarin Pharmaceutical Inc. As co-inventor of patents on exon skipping technology JD is entitled to a share of royalties.

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