Orthogonal analysis of dystrophin protein and mRNA as a surrogate outcome for drug development

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Aim: Detection of drug-induced dystrophin in patient muscle biopsy is a surrogate outcome measure for Duchenne muscular dystrophy. We sought to establish and validate an orthogonal approach to measurement of dystrophin protein and RNA in muscle biopsies. **Materials & methods:** Validated methods were developed for dystrophin western blotting, mass spectrometry, immunostaining and reverse transcriptase PCR of biopsy mRNA using muscle biopsy standards. **Results:** Both western blotting and mass spectrometry validated methods demonstrated good linearity, and acceptable precision and accuracy with a lower limit of quantitation at 1%. Immunostaining and reverse transcriptase PCR methods were shown to be reliable. **Conclusion:** The described orthogonal approach is sufficient to support measures of dystrophin as a surrogate outcome in a clinical trial.

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Duchenne muscular dystrophy (DMD) is a neuromuscular disorder with an incidence of 1/5000 newborn males, and results from loss of function mutations of the 2.3 million base pair *DMD* gene on the X chromosome, leading to loss of the dystrophin protein in muscle [1]. Most DMD patients show deletion mutations of one or more of the 79 exons, where the remaining exons can be spliced together into mRNA, but the transcript does not maintain the translational reading frame (out-of-frame mutation), and this leads to dystrophin-deficiency in patient myofibers. Becker muscular dystrophy (BMD) is a clinically milder disease caused by deletion of exons of the *DMD* gene, but the remaining exons are spliced into a mRNA that maintains the translational reading frame (in-frame mutation), leading to dystrophin of altered molecular weight and amount. A therapeutic approach pursued in DMD is to deliver oligonucleotide drugs that bind to an exon flanking a DMD patient's deletion mutation, causing skipping of the targeted exon in mRNA splicing and converting an out-of-frame mRNA transcript (DMD) to an in-frame mRNA transcript (BMD) [2]. If sufficient drug-induced *de novo* BMD-like dystrophin can be produced in DMD patient muscle, then a milder phenotype might be expected. Studies in dystrophin-deficient mice and dogs have shown strong proof of concept of this personalized mutation correction approach [3–5].

Clinical trials of targeting the skipping of exon 51 in DMD subjects have been reported for both 2'-O-methyl phosphorothioate drug chemistry and phosphorodiamidate morpholino oligomer chemistries [6–8]. According to the recent US FDA position on accelerated approval for Eteplirsen in 2016 [9], drug-induced dystrophin expression in patient muscle was considered to play a pivotal surrogate role in measuring the outcome in the phosphorodiamidate morpholino oligomer treatment targeting exon 51, as well as a committed postmarketing proof of efficacy.

Dystrophin is a large (427 kDa) and relatively low abundance protein (approximately 0.002% in striated muscle protein) that can be challenging to measure accurately and consistently by western blot methods [1]. Immunostaining of tissue sections with dystrophin antibodies is technically less challenging, but immunostaining is not typically considered a quantitative assay. Reverse transcriptase PCR (RT-PCR) of dystrophin mRNA isolated from patient biopsies can be used to determine unskipped (baseline) and skipped (drug-induced exon skipping) transcripts and can serve as a biomarker of drug mechanism of action. However, RT-PCR products are not considered a surrogate biomarker for drug approval in dystrophin replacement therapies. Mass spectrometry methods have been reported in a single method development paper [10], but have not been validated or utilized in exon skipping trials to date. An orthogonal approach, where multiple complementary molecular assays are brought to bear on the same patient muscle samples, should give a better picture of the success of dystrophin rescue, if the data are generated by blinded analysts and the resulting data from orthogonal assays consistent with each other.

A collaborative effort of five laboratories experienced in dystrophin measurements worked to harmonize dystrophin western blot and immunostaining methods [11]. Using six BMD patient muscle biopsies with varying levels of dystrophin, all five laboratories used the same western blot method on the same samples in parallel [12]. Three of the six biopsies studied showed levels of dystrophin most relevant to exon skipping trials (means relative to normal controls: 0, 0.4 and 11.4%), and these showed average percentage coefficient of variance (%CV) between the five laboratories of 222% (0.4% dystrophin biopsy), and 81% (11.4% dystrophin biopsy), considerably higher than suggested reliability of regulators of %CV of approximately 25% or less. The same biopsies were studied by three immunostaining methods, and the authors found that immunostaining was both more sensitive and reliable between labs and methods. In the same three low level dystrophin biopsies, immunostaining methods showed greater estimates of dystrophin: 0% blot biopsy showed 10.6% dystrophin-positive myofibers by immunostaining; 0.4% by dystrophin blot showed 9.6% dystrophin-positive fibers by immunostaining; 11.4% dystrophin blot showed 19.8% dystrophin-positive fibers by immunostaining. Western blot is generally considered more quantitative than immunostaining, and the FDA has echoed this opinion [9].

Here, we describe the method development, qualifications and validations for an orthogonal approach to dystrophin analyses, including dystrophin Western blot, mass spectrometry, immunostaining and RT-PCR in the context of a regulatory path for an exon skipping clinical trial.

Materials & methods

The described research was carried out by AGADA BioSciences, a clinical research organization in Halifax, Nova Scotia, Canada, with the mass spectrometry analysis performed by Altasciences, a contract research organization in Laval, Québec, Canada. Both AGADA and Altasciences were under contract by NS Pharma, Inc. (NJ, USA). Human muscle biopsies utilized for method development, method qualifications and validation studies were pre-existing de-identified archival frozen samples from DMD, BMD and non-DMD controls; none were treated with exon-skipping drugs. The DMD and BMD were obtained from Binghamton University tissue bank in Professor Eric P Hoffman's laboratory and were remaining material from molecular diagnostic services. The diagnostic biopsies were open biopsies. For non-DMD control samples, muscle samples were from either the Binghamton University tissue bank, or autopsy samples purchased from Cureline (CA, USA). Ethical approval for use of the archival de-identified muscle samples in this study was provided by an independent ethics institutional review board (Veritas, Quebec, Canada).

Antibodies

Antibodies used were: anti-dystrophin (ab15277, Abcam, MA, USA), anti-VCL (SPM227; Abcam), anti-ACTN2 (A7811; Sigma, MO, USA), anti- α -SPTA1 (ab139403; Abcam) and anti-LAMA2 (MAB1922; Millipore, MA, USA).

The anti-dystrophin antibody (ab15277) is an affinity-purified rabbit polyclonal that has been used in western blot and immunohistochemistry, and tested in mouse, rat, dog and human, cited in 155 publications, and independently reviewed in 20 reviews. Importantly, this same antibody has been used by international consensus working groups on dystrophin measurement in human muscle biopsies [11]. Further evaluation done by AGADA included demonstration of high specificity (no detectable bands in both human and dog biopsy samples lacking dystrophin due to gene mutation), and linearity of quantitation of mixed DMD and normal human samples (Method development below).

Anti-VCL (SPM227) and anti- α -SPTA1 (ab139403) antibodies were utilized only in method development and were not included into the final methods. Anti-ACTN2 (A7811) antibody was utilized in the final method and the A7811 reagent is a monoclonal antibody (IgG1 from ascites) against the *ACTN2* gene product (sarcomeric α -actinin). It has been used in western blot and immunohistochemistry in multiple species (including human), cited in 499 peer-reviewed publications. This antibody has been also used by the international consensus working group [11]. Anti-LAMA2 (MAB1922) antibody has been used in the immunohistochemistry and tested in human, monkey and rabbit, and cited in 38 publications.

Western blotting

Initial sample processing focused on maximizing solubilization of full-length (intact) dystrophin from dense skeletal muscle tissue. For solubilization, we increased the efficiency of solubilization and reduced likelihood of proteolysis and physical breakage of the large dystrophin protein by using cryosections. Starting material was ≈ 50 frozen muscle biopsy cryosections (10 μ m thickness), placed into pre-chilled (-40°C) 1.5 ml centrifuge tubes and stored at -80°C until solubilization. For solubilization, 100 μ l room temperature (RT) SDS tissue solubilization buffer ([13]; 10% sodium dodecyl sulfate, 0.1M Tris pH 8, 15 mM dithiothreitol and Bromophenol Blue) was quickly added to the frozen sections, and the samples then vortexed for 40 s at RT, heated at 90°C for 2 min and then centrifuged to pellet nonsoluble proteins for 10 min. Protein concentration of the supernatant was performed using BCA protein assay kit (Pierce, MA, USA). Solubilized samples were stored at 4°C and used within 48 h to eliminate freeze–thaw cycles and minimize degradation of the dystrophin protein.

For western blotting, 50 μ g of total proteins was loaded per well, using NuPAGE 3–8% Tris-Acetate Midi gels (ThermoFisher Scientific, CA, USA). A standard curve for dystrophin content was included for each gel. The samples for the standard curve were a mixture of non-DMD control biopsy sections (five different normal control biopsies, each previously shown to have equal amounts of dystrophin [$\sim 100\%$ dystrophin]), and a DMD patient biopsy (previously shown to have no detectable dystrophin). Gels were electrophoresed with Tris acetate running buffer for 75 min at 150 V. The transfer of the protein to nitrocellulose membrane was performed using wet transfer (submerged) for 4 h at 0.3 Amps with constant stirring. Following the transfer, the nitrocellulose membrane was wiped clean of any acrylamide residue in clean running buffer and then dried overnight. The post-transfer gel was stained with Coomassie Blue for detection of residual MYH proteins – digital scans of the MYH proteins in the post-transfer gel were then used as one of the myofiber-specific proteins for normalization of dystrophin protein content.

For immunological detection of dystrophin and control proteins, the dried nitrocellulose membrane was cut with scissors into a high molecular weight range (for dystrophin detection; ab15277, Abcam, MA, USA; 1:300), and low molecular weight range (for VCL [SPM227; Abcam; 1:1000], ACTN2 [A7811; Sigma, MO, USA; 1:500] or α -SPTA1 [ab139403; Abcam; 1:1000]). Both sections of nitrocellulose membrane were then blocked in 5% skim milk in phosphate buffered saline with 0.1% Tween 100 (PBST) for 1 h at RT, and then probed with primary antibodies diluted in the same 5% milk PBST buffer for 3 h with constant shaking. The membranes were then washed three-times for 10 min at RT and reblocked with 5% skim milk for 5 min on the shaker. The secondary antibody (horse radish peroxidase [HRP]-conjugated) was added to the blots for 1 h. Membranes were washed again with PBST three-times for 10 min, each at RT on the shaker. Enhanced chemiluminescence detection reagents (GE Healthcare, UK) were added and chemiluminescent images of the bands on each membrane were taken using ChemiDoc XRS+ system (Biorad, CA, USA).

Quantitation of intensity of blots and gels were done using Image Lab software (Bio-Rad, CA, USA, v 5.2.1). Results were calculated as a percent of normal (100% dystrophin), using the 0–100% standard curves on the same membranes, and normalized to both MYH (Coomassie Blue gel) and ACTN2 (low molecular weight nitrocellulose) on the same gels. Triplicate gels were done for each set of samples by two different analysts for the validation study.

FDA has published guidelines for bioanalytical method validation in the context of drug development (www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf). Included in these guidelines are the development of prespecified acceptance and rejection criteria so that each experiment meets thresholds for inclusion in data analyses. We developed acceptance and rejection criteria for each method during method development. The following acceptance and rejection criteria for western blot were prespecified as follows:

- Standard curves must show fit to a polynomial curve;
- One percent normal control is higher than DMD-only lane;

- The ACTN2 and MYH loading controls are less than 50% of the relative standard deviation of average;
- If discordant data are obtained (>30% of determined value), the analysis is viewed as a failure, and would be repeated;
- The lower and upper limit of quantitation should not exceed 40 and 30%, respectively of the %CV and the accuracy within 70 and 40%, respectively of the nominal concentration. The accuracy was calculated as follows:

$[\text{Mean of the replicates (\% dystrophin)} - \text{expected value (\% dystrophin)}] / \text{expected value} \times 100\%$

The values within the acceptance and rejection criteria were predefined values as per FDA guidelines. The FDA guidelines recognize that there are broader %CV observed for lower levels of a biomarker, and narrower %CV for higher levels of a protein.

Mass spectrometry

For mass spectrometry sample analysis, 70 serial cryosections (10 μm thickness) were prepared from each of the human muscle biopsies and directly placed into a prechilled 1.5 ml microcentrifuge tube on dry ice. Total protein extraction from the muscle samples was performed by vortexing with radio immuno precipitation assay (RIPA) buffer containing protease and phosphatase inhibitors. Protein concentration was determined using BCA assay. A total of 50 μg aliquots from each extract were spiked with 12.5 μg of stable isotope labeled protein extract prepared from stable isotope labeling with amino acids in human myotube (SILAC), leading to ^{13}C and ^{15}N labeling at lysine and arginine in all proteins [14]. The myotubes were labelled for 12–15 days before they were detached from the flask using Trypsin-EDTA (Millipore, MA, USA). The pellets were lysed in RIPA buffer (Sigma, MO, USA) with protease inhibitor cocktail and the protein concentration was quantified using Pierce™ BCA protein assay kit. Using this method, 99% of the dystrophin was labelled with heavy isotopes. The mixture was then fractionated by gel electrophoresis using 3–8% Tris-Acetate gel. After gel electrophoresis, the band corresponding to dystrophin size between 268 and 460 kDa marker was excised and in-gel digested with trypsin gold (mass spectrometry grade, Promega, WI, USA). Peptides were extracted from the gel piece using 25 mM ammonium bicarbonate, acetonitrile and two changes of mixture of 10% formic acid and acetonitrile, dried by vacuum centrifuge and sent to Altasciences (Laval, QC) for mass spectrometry analysis.

Qualitative analysis

To identify dystrophin peptides, as well as background proteins present in the muscle extracts, a Top10 data-dependent LC–MS/MS method was used on the Q Exactive™ Plus coupled with the EASY-Spray™ source and the Dionex 3000 Ultimate RSLCnano HPLC system (ThermoFisher Scientific). Dried total peptide from in-gel digested samples were resuspended in 20 μl solution of 2% acetonitrile and 0.05% TFA and 3 μl injected through an autosampler onto a C18 analytical column (Acclaim PepMap C18, 75 μm x 15 cm, 3 μm , 100 Å). A 120 min linear gradient (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile) was used at a flow rate of 300 nl/min. The MS raw data were queried using Proteome Discoverer v2.1 (ThermoFisher Scientific) against the human FASTA database at high confidence level with two allowed trypsin miscleavages, 10 ppm precursor mass tolerance and 0.02 Da product mass tolerance.

Quantitative analysis

For quantitative analysis, dried samples were redissolved in 40 μl of 2% acetonitrile and 0.1% TFA in LC–MS grade water. A 20-min linear gradient from 1 to 35% (A: 0.1% formic acid in water; B: 0.1% formic acid in acetonitrile) was used at a flow rate of 500 nl/min. The Q Exactive™ Plus mass spectrometer was operated in positive, parallel reaction monitoring mode. The masses of endogenous (light) and stable isotope labeled (heavy) dystrophin and filamin C peptides were added to the inclusion list. Two dystrophin peptides and two filamin C peptides were selected for quantitation by LC–MS/MS. The quadrupole isolation window was set at 1.0 m/z and the Orbitrap resolution at 17,500.

To assess LC–MS/MS system suitability, Pierce™ Peptide Retention Time Calibration Mixture (ThermoFisher Scientific) was injected at the beginning and at the end of each batch run. Overall system sensitivity, mass accuracy and LC retention times were monitored.

For data processing, LCquan 3.0 software (ThermoFisher Scientific) was used to generate dystrophin and filamin C peptide peak areas by summing the peak areas of the corresponding product ions. Peptide ratios were calculated by dividing the peak areas of light peptide with the peak area of corresponding heavy peptide. Using the individual

peptide ratios, an average peptide ratio of dystrophin and filamin C was then generated in an Excel template. The final normalized dystrophin area ratio was calculated by dividing the dystrophin area ratio with the corresponding Filamin C area ratio.

Immunostaining

Acceptance and rejection criteria were defined through method development and were established with thresholds that were felt to be sufficient for downstream analyses of clinical trial biopsies. Cryosections (10 μ m) of frozen muscle biopsies were stained with hematoxylin and eosin to assess the following prespecified acceptance and rejection criteria:

- Less than 10% of myofibers can show evidence of freeze artifact:
 - Freeze artifact must be observed as nonstaining (noneosinophilic) ‘holes’ in a myofiber;
 - The presence of freeze artifact complicates interpretations of immunostaining patterns;
- More than 80% of myofibers must be visible in cross section:
 - Myofibers should be quantified in cross section;
 - Longitudinal myofibers are more challenging to count, and therefore must be excluded from quantitation;
- More than 50% of the muscle section area must be comprised of myofibers:
 - The analytical samples will only be examined if they contain predominantly muscle fiber (skeletal muscle) cells, and no greater than 50% fibrofatty connective tissue;
- More than 200 muscle fibers per section.

For those samples meeting acceptance criteria, 10 μ m sections were mounted on SuperFrost Plus slides and stored at -80°C until the staining was performed. Slides were thawed and blocked for 30 min with 5% horse serum (HS) in phosphate buffered saline (PBS) at RT. Anti-dystrophin antibody (ab15277, Abcam; 1:500) and anti-LAMA2 (Millipore, MA, USA; 1:5000) or anti-SGCA (Iowa Developmental Studies Hybridoma Bank, IA, USA; 1:4) were delivered to the sections in the presence of 5% HS and incubated at RT for 2 h. All slides were then washed three-times with 1x PBS 5% HS at 5-min intervals. The secondary antibody Alexa Fluor 594 AffiniPure Goat Anti-Rabbit (Cedarlane, ON, Canada; 1:500) and Alexa Fluor 488 Goat anti-mouse IgG1 (ThermoFisher Scientific; 1:500) was then added to the sections in the presence of 5% HS and left for 15 min at RT. The slides were washed three-times with 1x PBS + 5% HS at 5-min intervals. Fluoroshield with DAPI (Sigma) was added to all tissue sections before the coverslip was added.

All slides were imaged at 20 $\times$ magnification using the Leica Aperio Versa 8 scanner (Leica, IL, USA) and then stored at 4°C . Samples were blinded to the analysts analyzing the samples. Two analysts unaware of the biopsy identity analyzed each sample. The number of dystrophin positive fibers normalized to the number of LAMA2 positive fibers was expressed as percent dystrophin positive fibers. Quantitation of positive dystrophin muscle fibers is broken down into three categories based on the physical integrity of the plasma membrane and immunostaining intensity: complete (100%) dystrophin positive fiber with no breaks and strong immunostaining; dystrophin positive fiber with partial membrane break, incomplete plasma membrane staining, or both; and dystrophin positive fiber with a major break ($\leq 50\%$). A membrane break $> 50\%$ is considered a negative fiber. Each category is counted as positive and recorded within Aperio ImageScope software (Leica, IL, USA, v. 12.3.2.8013).

The intensity of dystrophin-positive fibers normalized to LAMA2 positive fibers or SGCA positive fibers were also calculated. The threshold for the analysis was set such that the BMD sample had approximately 80% dystrophin-positive fibers as internal control. Once the threshold was set, the same threshold was used to analyze all other samples for dystrophin-positive fibers count.

Reverse transcriptase PCR

RNA was isolated from approximately 30 frozen cryosections (30 μ m) of non-DMD biopsies placed in prechilled, RNase-free 1.5 ml centrifuge tubes as there is no suitable DMD and BMD biopsies. Tissue sections were stored at -80°C until use. Total RNA was isolated from each sample using RNeasy Micro Kit (QIAGEN, Hilden, Germany) as per manufacturer recommendations. Briefly, the sections were lysed in buffer RLT in the presence of β -mercaptoethanol and ethanol added to precipitate the RNA. The total RNA was then added to the spin column and subjected to DNase digestion and washes. The RNA was then eluted in RNase-free water. All RNA

was aliquoted before freezing at -80°C to prevent repeated freeze–thaw cycles and potential physical shearing of the large dystrophin mRNA (14 kb).

RNA quality was measured using the Experion Bioanalyzer to determine RNA concentration and quality (RNA Quality Index [RQI]) using the acceptance criteria below:

- RNA concentration $> 100\text{ ng}/\mu\text{l}$;
- RQI is > 7 (RQI is based on a normalized ratio of 28 S and 18 S ribosomal RNA bands, with a score of 10 being the most intact).

Once the RNA quality passed the acceptance criteria, the samples were then subjected to RT-PCR using QIAGEN OneStep RT-PCR kit (QIAGEN, Hilden, Germany) with three distinct primer sets designed against different exons of the dystrophin RNA transcript where the reverse primer was kept constant across all three primer sets (44F + 54/55R, 46F + 54/55R and 48F + 54/55R). Total RNA at 50 ng was used per reaction in a 25 μl PCR reaction volume with the following thermal profile: 1 cycle of 50°C for 30 min, 95°C for 15 min, 30 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, 1 cycle of 72°C for 7 min. The resulting RT-PCR products were then diluted 1:5 before loading onto the Experion DNA 12K Analysis Kit for detection. Each DNA chip contained three samples (non-DMD, ran in duplicate). Acceptance/rejection criteria were defined during method development and were felt to enable later quantitative analyses of patient muscle biopsies from clinical trials. The acceptance/rejection criteria were prespecified for method validation. All samples passed the acceptance criteria for both band intensity of over 50% of the total band intensity and the amplicons detected on the gel were all within $\pm 25\%$ of the predicted RT-PCR product size.

The following primer sequences were used for the RT-PCR, and were synthesized by IDT, Coralville, IA:

Forward primers: 44F: 5'- CCTGAGAATTGGGAACATGC –3', TE 50 pmol/ μl

46F: 5'- AACCTGGAAAAGAGCAGCAA –3', TE 50 pmol/ μl

48F: 5'-CCAAGAAGGACCATTGACG –3', TE 50 pmol/ μl

Reverse primer: 54/55R: 5'-TCTCGCTCACTCACCCCTTTT-3', TE 50 pmol/ μl .

Results

Western blotting method development

DMD patient muscle shows fibrofatty replacement of myofibers as the disease progresses. As dystrophin is expressed in myofibers, and not fibrofatty tissue, accurate assessments of dystrophin normalize to myofibrillar proteins (rather than total protein). For method development, we tested four control proteins – VCL, α -SPTA1, ACTN2 and MYH. Three of these were detected on the same western blot as the dystrophin protein (VCL, α -SPTA1, ACTN2) and one was on post-transfer gels stained with Coomassie Blue (MYH).

Four western blots were run, each with a dystrophin standard curve by mixing of non-DMD control and DMD samples (0, 2, 10, 25, 100% of non-DMD control biopsies), with additional lanes for two quality control (QC) samples prepared by mixing non-DMD and DMD samples to obtain approximately 5% dystrophin level of normal, and two additional non-DMD control biopsies (Figure 1 & Supplementary Figure 1). The standard curve and QC samples were prepared using the same non-DMD samples (five mixed normal controls), although two different dystrophin-negative DMD biopsies were used. The nitrocellulose was cut into four regions and stained for dystrophin and the three control proteins (VCL, α -SPTA1, ACTN2). Post-transfer gels were stained with Coomassie Blue for MYH. Chemiluminescence signal was digitally captured for all bands, and the MYH gel digitally scanned by light transmittance.

Dystrophin bands of the standard curve (2, 10, 25 and 100%) were then normalized to each of the four control proteins, and %CV calculated (Figure 2). As dystrophin signals were kept constant across the four normalizations, the %CV reflects the precision of each control protein for varying levels of dystrophin upon test/re-test, as a function of dystrophin level (lower limit of quantitation [LLOQ; 2% normal levels] vs higher limit of quantitation [HLOQ; 25% normal levels]). This showed that myosin heavy chain, α -actinin and α -spectrin showed similar %CV (44.5–61% at LLOQ [2%], and 38–43% at HLOQ [25%]). Vinculin showed substantially poorer precision, with higher %CV at all dystrophin measures (63% at LLOQ [2%], and 86% at HLOQ [25%]).

QC samples ($\sim 100\%$ and $\sim 5\%$ dystrophin) were tested from two non-DMD biopsies ($\sim 100\%$ dystrophin), and two samples with low levels of dystrophin ($\sim 5\%$ dystrophin) obtained by mixing non-DMD and DMD samples. Normalization to each of the four control proteins, and determination of dystrophin content of each

Figure 1 Optimization of myofiber control protein. Shown are four parallel western blots detecting dystrophin (D; 427 kDa), ACTN2 (A; 100 kDa), VCL (V; 124 kDa), α -SPTA1 (S; 280 kDa). The post-transfer acrylamide gel for each western blot was stained with Coomassie Blue to detect residual MYH proteins (M; 200 kDa). A standard curve of normal control mixed with DMD was included in each gel (0, 2, 10, 25, 100% dystrophin). QC samples prepared by mixing non-DMD and DMD samples to produce low dystrophin content are shown (5% [1]; 5% [2]) as well as additional normal controls (Normal 2, Normal 3). Dystrophin signals were normalized to each control protein, and % coefficient of variance for repeated measures determined. DMD: Duchenne muscular dystrophy; QC: Quality control.

Figure 2. Determination of precision of four normalization proteins in dystrophin standard curve. CV: Coefficient of variance; DMD: Duchenne muscular dystrophy.

Figure 3. Testing of quality control samples from normal controls (~100% predicted dystrophin) and low-level dystrophin (~5% predicted dystrophin). Normal 1 is back calculated (~100% dystrophin). CV: Coefficient of variance; QC: Quality control.

sample relative to the standard curve was done (Figure 3). Similar to the data obtained for the standard curve, both MYH and ACTN2 appeared to have lower %CV across all the samples compared with VCL and α -SPTA1 (Figure 3). In addition, the sensitivity was also significantly improved using high SDS solubilization buffer. The mean absorbance value for the 2% dystrophin on the standard curve was approximately twice that of the 0% DMD-only lane. On the other hand, there was no difference in the mean absorbance between 2% dystrophin and 0% DMD-only lane when the solubilization was performed using RIPA buffer (data not shown).

This analysis showed that normalization to α -SPTA1 led to higher measured levels of dystrophin relative to other controls (two- to fourfold increase), as well as a higher %CV for most values, suggesting that α -SPTA1 was more variable between biopsies. Normalization to ACTN2 tended to underestimate dystrophin levels relative to the other controls, but also showed low %CVs (18–45% for HLOQ [$\sim$ 100%]; 64–68% for LLOQ [$\sim$ 5%]). Normalization to MYH showed similar low %CVs as ACTN2, while the dystrophin levels approached the average of all controls (and the predicted or anticipated percentages).

In conclusion, these analyses showed that both ACTN2 and MYH performed well as normalization controls, with relatively good precision at both LLOQ and HLOQ. Of these, accuracy regarding finding the predicted levels of dystrophin was better for MYH, with determined dystrophin levels more similar to predicted levels.

There may arise a concern that the post-transfer MYH protein measures were not isoform-specific. Specifically, there are multiple specific types of MYH that co-migrate in the gel and are thus quantitated by the post-transfer

Figure 4. Western blot images of standard curve and quality control samples after normalization to MYH and ACTN2 from Analyst 1 (A) and Analyst 2 (B). Shown are representative blot images of the 0–100% dystrophin standard curve and QC samples. Dystrophin standard curve plotted after normalization to ACTN2 and MYH are shown. QC: Quality control.

stain. The ACTN2, on the other hand, detects a single protein species. Due to the differences in accuracy of the two measures, we decided to carry both MYH and ACTN2 normalization controls forward into subsequent validation studies.

Western blotting validation

The validation protocol was established to reflect the final clinical sample analyses in the context of clinical trials. Each experiment (western blot gel) included a standard curve, as described in Method Development. However, as the lanes on each gel were limited and the standard curve must bracket the anticipated results of the clinical samples, our validation included a five point (sample) standard curve (0, 2, 10, 25, 100%) and four clinical samples (QC samples). This was later modified to include a lower sample to better bracket anticipated clinical results (0, 1, 2, 10, 25%). For method validation, two independent analysts carried out this validation study (Analyst 1; Analyst 2), each running three replicate western blots ($n = 6$ gels total). Sectioning, solubilization, quantitation and all steps of the validation plan were done independently by the two analysts on separate days.

All gels fulfilled all acceptance and rejection criteria for the QC samples (Figure 4, Table 1 & Supplementary Figure 2). The R^2 values of standard curves were between 0.9991 and 0.9997. All gels met the acceptance criteria of higher dystrophin value in the 1% QC samples relative to no dystrophin (DMD), with the quantified values ranging between 1.19 and 3.01% (normalized to ACTN2) and between 1.36 and 2.35% (normalized to MYH). The ACTN2 and MYH loading controls were less than 50% of the relative standard deviation of average.

In this validation study, normalization to either ACTN2 or MYH yielded similar level percent dystrophin for both analysts. As for the lower and upper limit of quantitation and accuracy of the nominal concentration, dystrophin

Table 1. Validation results of western blot for quality control samples for Analyst 1 and 2.

QC samples	Analyst 1				Analyst 2			
	Mean	SD	%CV	Accuracy%	Mean	SD	%CV	Accuracy%
Derived % of dystrophin normalized to α-actinin								
20%	16.0	2.2	13.9	20.2	19.2	7.1	37.0	4.0
10%	7.1	2.6	36.0	28.9	8.0	2.4	30.5	20.3
5%	3.4	1.5	44.2	32.0	4.4	1.3	29.1	11.7
1%	1.4	0.3	22.7	40.3	2.3	0.8	36.6	128.0
Derived % of dystrophin normalized to myosin heavy chain								
20%	14.8	2.3	15.6	26.0	15.6	3.5	22.5	22.1
10%	7.1	1.7	23.4	29.8	8.1	0.8	10.2	19.4
5%	3.9	0.7	16.6	21.7	4.0	0.6	13.9	19.5
1%	1.7	0.6	32.8	69.7	2.1	0.4	19.3	106.7

CV: Coefficient of variance; QC: Quality control; SD: Standard deviation.

normalized to MYH met CV criterion (%CV: 15.6 and 22.5% for HLOQ; 19.3 and 32.8% for LLOQ) fulfilled accuracy criterion in three of four measures (single 1% measure was 36.7% outside tolerance for accuracy for LLOQ). On the other hand, dystrophin normalized to ACTN2 did fulfill the lower and upper limit of quantitation as well as the accuracy for Analyst 1 but %CV for the upper limit of quantitation was just outside the tolerance range by 7% and the accuracy by 58% for the LLOQ for Analyst 2 (Table 1).

Overall, the method had been validated and that MYH appeared to be a better internal control compared with ACTN2, with lower %CV for LLOQ and HLOQ compared with ACTN2.

Mass spectrometry method development

Nanoflow LC–MS/MS analysis of normal muscle extracts led to the identification of 160 dystrophin peptides corresponding to 50% sequence coverage. Additionally, several muscle specific proteins were observed including filamin C with 106 peptides corresponding to 50% sequence coverage. This myofiber-specific protein was selected as an internal control for protein content normalization and precise quantitation of dystrophin. Based on these results, 15 custom dystrophin peptides were synthesized (New England Peptide, MA, USA) and used for method development and surrogate peptide selection. The best candidates were determined based on chromatographic behavior, MS ionization and peptide fragmentation. Two dystrophin peptides, IFLTEQPLEGLEK and LLQQF-PLDLEK, as well as two filamin C peptides, SPFVVNVAPPLDLSK and VAVGQEQAQFSVNTR, were selected for dystrophin quantitation (Supplementary Table 1).

Technical replicates of normal muscle extracts (50 μ g) from the same biopsy injected six consecutive times showed very good precision (%CV = 5.19 and 4.79 for dystrophin peptides 1 and 2). To determine the LLOQ, normal muscle extracts diluted with DMD muscle extracts were analyzed and the method showed very good sensitivity down to 1% of normal dystrophin level (LLOQ; Supplementary Figure 3). The quantity of SILAC extracts to add to 50 μ g of muscle extracts prior to gel electrophoresis was determined by analyzing different amounts to verify the dystrophin expression levels and, more importantly, the labeling efficiency for every new SILAC batch preparation.

Mass spectrometry method validation

To prepare calibrants and QCs, 50 μ g aliquots from each muscle extract were spiked with 12.5 μ g of stable isotope labeled proteins prepared from SILAC cultured human myotubes. For method validation, two Coomassie Blue gels were prepared on two separate days. To limit gel-to-gel extraction variability, calibration standards and QCs from the same precision and accuracy batch were run on the same gel. Each gel was comprised of a standard curve (0, 1, 3, 10 and 25% dystrophin) and QC samples (2, 7 and 15% dystrophin) prepared by mixing non-DMD control samples (mixture of five non-DMD biopsies) and a DMD sample (mixture of two DMD biopsies). The absence of dystrophin in the DMD biopsies used for MS analysis was verified by western blot. In addition, a blank sample (0% dystrophin without IS) and an IS-only sample (SL) were included in both gels. In-gel digested samples from the two gels were analyzed by LC–MS/MS on two separate days, using two different lots of analytical columns and different lots of solutions.

Figure 5. Dystrophin standard curves and quality control plots for Gels 1 and 2. QC: Quality control; STD: Standard.

Table 2. Back-calculated concentrations of dystrophin and curve parameter for Gels 1 and 2.

Sample type	Gel 1		Gel 2	
	Calculated concentration	Difference from nominal	Calculated concentration	Difference from nominal
Calibrant samples (% dystrophin)				
1.0	1.2	21.8%	1.1	8.0%
3.0	2.4	-18.8%	2.9	-3.8%
10.0	9.3	-7.3%	9.3	-7.3%
25.0	26.1	4.3%	25.8	3.1%
QC Samples (% dystrophin)				
2.0	2.5	23.6%	2.0	-2.1%
7.0	6.8	-3.1%	7.1	1.5%
15.0	15.4	2.9%	15.8	5.6%
R ² value	0.9911		0.9969	

QC: Quality control.

The results showed no detection of dystrophin or filamin C in the analysis of the empty lane indicating the absence of cross contamination between gel lanes. Furthermore, no dystrophin peptides were detected in blank samples. Low levels of dystrophin (below 1%) were observed in blank samples containing the internal standard due to the contribution of unlabeled dystrophin from the preparation of stable isotope labeled human myotubes.

The calibration curves were fitted using a weighted $1/x$ linear regression giving R^2 values of 0.9911 and 0.9969 for both gels, respectively (Figure 5). The inter-assay precision values were determined at 15.7% for low QC, 3.1% for mid QC and 1.8% for the high QC. All back-calculated calibrant and QC values are within the acceptance criteria of $\pm 30\%$ from nominal using the normalized mean ratio of two dystrophin peptides for quantitation and with the subtraction of the 0% standard (Table 2).

Stability of the samples in injection medium was assessed by re-injecting the precision and accuracy batch from gel 2 after storage at 10°C (autosampler) and at 4°C between re-injections. The combined stability period was proven for 20.4 h at 10°C and 14 days at 4°C (Supplementary Table 2). This stability timeframe ensures ample time for batch re-injection if warranted.

Immunostaining method development

The samples analyzed for immunostaining method development and validation included non-DMD biopsy (100% dystrophin controls), DMD biopsies (one with revertant myofibers, and one without revertant fibers), and three manifesting carrier biopsies (somatic mosaics for dystrophin expression [15,16]. The manifesting carrier samples were used as models for dystrophin gene rescue in DMD patients and to count percentage of dystrophin-positive myofibers in a cryosection.

Figure 6. Co-immunostaining of muscle biopsies for dystrophin (left) and LAMA2 (right). (A) Non-DMD control. (B) DMD biopsy with no revertants. (C) DMD biopsy with isolated revertant fibers. Bar = 300 μ m. DMD: Duchenne muscular dystrophy.

Two immunostaining protocols were done in parallel. The first protocol used co-staining of dystrophin with LAMA2. LAMA2 is an abundant component of the myofiber basal lamina and was used to define all myofibers in cross section in a cryosection of a biopsy. The second protocol used co-staining of dystrophin and SGCA (a dystrophin-associated glycoprotein). This second protocol enabled co-localization of dystrophin and SGCA to define expected membrane localization of dystrophin, and also studied restoration of the dystrophin-associated glycoprotein complex.

One analyst processed the biopsies for the two immunostaining protocols in parallel (dystrophin + LAMA2; dystrophin + SGCA). Digital images were then acquired and two analysts, different from the analysts that performed the immunostaining protocol, performed quantitation of myofiber analyses. Each analyst chose a complete cryosection and counted all myofibers in that cryosection. All muscle biopsy sections that were to be used for method development passed pre-defined acceptance/rejection criteria in materials and methods section.

Dystrophin co-stained with LAMA2 or SGCA showed expected patterns, with all myofibers stained for LAMA2 and only dystrophin-positive fibers showed increase SGCA staining in non-DMD, DMD and DMD with revertants (Figure 6 & Supplementary Figure 4). Also as expected, dystrophin immunostaining showed all myofibers to be positive in non-DMD control, with only rare revertant myofibers stained positively in the one DMD sample. Quantitation of digital images of entire cryosections was independently scored by two analysts. This showed reasonable agreement of the two analysts regarding the percentage of dystrophin-positive myofibers for both protocols in the three control biopsies studied (non-DMD controls [99.4 vs 97.8%], DMD with revertants [1.3 vs 1.3%], and DMD without revertants [none detected]; Supplementary Table 3). Measuring the intensity level of dystrophin normalized to SGCA showed the abundant level of dystrophin in the non-DMD controls (4.0 vs 4.5), low level of dystrophin in the DMD samples with revertant fibers (0.85 vs 0.71) and none-detected in the DMD samples (Supplementary Table 3).

In the three experimental samples, the level of dystrophin-positive muscle fibers as analyzed by counting the number of fibers and measuring the intensity of dystrophin normalized to LAMA2 and dystrophin normalized to SGCA showed considerable variation between samples (Table 3 & Supplementary Figures 5 & 6). This was not

Table 3. Analysis of the fiber count for dystrophin-positive myofibers normalized to either LAMA2 or SGCA for the three female carrier samples. The values in the parentheses are numbers of myofibers counted for LAMA2 and intensity of the stain for SGCA.

Parameters measured	Female carrier (A)		Female carrier (B)		Female carrier (C)	
	Analyst 1	Analyst 2	Analyst 1	Analyst 2	Analyst 1	Analyst 2
Dystrophin positive fiber count normalized to LAMA2 (%)	73.2% (1787/2443)	70.5% (1512/2146)	61.3% (1040/1697)	59.4% (951/1601)	95.4% (864/906)	81.0% (597/737)
Quantity of dystrophin normalized to SGCA (intensity)	1.1 (0.18/0.15)	1.1 (0.16/0.14)	1.4 (0.20/0.14)	1.6 (0.22/0.14)	1.0 (0.11/0.11)	1.0 (0.12/0.12)

surprising when taking the nature of the samples into consideration. The muscle of manifesting carriers contained varying levels of dystrophin protein (normal to absent) and it varied between carriers.

Immunostaining method optimization

In the immunostaining method development, there was no reference tissue to set the gain of analysis. This is an integral component of the analysis in order to ensure that the analysis of the immunostaining is consistent and unbiased across samples tested over time. To address this, we chose a muscle biopsy from a BMD patient showing 20% dystrophin by western blot (data not shown) and used cryosections as a reference sample in this method validation study. The validation for the immunostaining consisted of testing normal control, DMD with revertants (as test samples) and the BMD sample (as digital normalization sample). The digital gain (1.75) for all images was set for the BMD sample so that approximately 80% of the muscle fibers showed positivity for dystrophin (Supplementary Figure 7). Images were then analyzed by two analysts to determine the proportion of dystrophin-positive fibers (Supplementary Table 4). Interestingly, intensity measurement ratio for dystrophin to LAMA2 did not achieve the value of 1 or closer for any of the samples analyzed. Since the main purpose for LAMA2 staining is for the quantitation of the total muscle fibers number in order to normalize the dystrophin value, the total fiber count should be considered rather than the intensity. On the other hand, the SGCA staining intensities ratio were closer to the value of 1. Both analysts achieved similar data. This suggests that the level of dystrophin correlate to the level of α -sarcoglycan. Overall, the immunostaining method had been optimized to detect the clinical samples that has less than 20% dystrophin recovery after exon skipping gene therapy.

RT-PCR method qualification

The method for RT-PCR was adapted from previous published report [17]. The purpose of this qualification was to demonstrate performance of an established method for detecting dystrophin mRNA by RT-PCR using gene-specific primers.

RNA was extracted from three non-DMD muscle biopsies samples by two analysts on two separate days. RNA was isolated from each biopsy once by each analyst, and RNA processing done in duplicates for each RNA by each analyst. Initial quality and concentration of RNAs were determined using Experion RNA Eukaryotic StdSens analysis. Each RNA sample was assessed on concentration of RNA (ng/ μ l) and the quality of RNA as described by the RQI value (ratio of 28 S and 18 S rRNA bands; range 1–10). All RQI values were >9.7 indicating excellent RNA quality [DATA NOT SHOWN].

Both analysts identified full length RT-PCR bands as expected for each primer set for each RNA. Expected bands were seen in all samples, with the expected bands representing 100% of bands in the electrophoretogram (i.e., highly specific; Supplementary Table 5). Regarding observed molecular weight of the RT-PCR products versus expected size, the largest RT-PCR product of 1.6 kb (44F/[54/55R]) showed the highest deviation by both analysts (10.5–13.7%) as might be expected due to loss of resolution at higher molecular weight bands. The protocol consistently achieved good performance in muscle biopsies in terms of RNA quality and fidelity of PCR products.

Discussion

Recently approved Eteplirsen claimed the increased dystrophin in DMD patient muscle biopsy and the regulatory authorities accepted it as a surrogate outcome measure [9]. Therefore, validation methodology for dystrophin rescue

would become more critical. In this report we describe the development of an orthogonal approach to dystrophin protein and RNA measures in patient muscle biopsies in support of exon skipping clinical trials in which western blot of dystrophin protein is the primary outcome measure, with mass spectrometry analysis, RT-PCR of dystrophin RNA and dystrophin protein immunostaining as orthogonal measures for assurance. Histological morphometry (myofiber sizes) was not an outcome of the clinical trials, and thus not studied as part of the methods development and validations. The goal of this study was to provide a prototype for orthogonal methods that could support demonstration of successful dystrophin recovery in DMD patient muscle as a surrogate outcome in clinical trials.

The western blot methods described here are similar to previous recent reports [8,11,13]. Differences between our method and previous reports were our use of very high SDS solubilization buffer (10% SDS) to achieve more consistent solubilization of dystrophin. We also used only freshly solubilized samples from cryosections, with no storage of samples as lysates. This was to avoid freeze–thaw cycles that can physically shear the large dystrophin protein, and thus decrease detection of full-length protein on western blots. The sensitivity of our approach was 1% of normal dystrophin levels with good precision in a formal validation study (%CV between 19 and 37% for low level of quantitation; acceptable predefined value for %CV in this study was no greater than 40%; Table 1). A recent report focused on the sensitivity and reliability of a newer technique, capillary western immunoassays and the levels of dystrophin seen in a series of normal controls, DMD and BMD patient muscle biopsies [18]. This method had advantages over gel-based western systems in lower sample requirements (~1 µg total protein), excellent sensitivity (<1% normal levels), strong linearity of standard curves diluting normal muscle with DMD extracts ($r^2 > 0.99$) and good sensitivity and reliability for low levels of dystrophin (<1% dystrophin, %CV ~10%). An alternative quantitative method that has been reported to show high sensitivity and reliability in detecting dystrophin is ELISA [19]. We did not study ELISA in part due to loss of molecular weight assessments of smaller dystrophin proteins induced by exon skipping (Becker-like), and in part due to reported challenges in standardizing and validating ELISA assays for dystrophin [20].

Another aspect of method development was the use of normalization controls for myofiber content of the tissue. DMD is a progressive disease, with age-related replacement of myofibers in muscle by fibrofatty connective tissue. As dystrophin is expressed in myofibers, and not fibrofatty connective tissue, normalization to myofiber content of a sample instead of the more typical total protein content is generally felt to be a more relevant measure in patient muscle biopsies. In comparing four normalization controls (α -SPTA1, VCL, ACTN2, MYH) we found that the precision measured by %CV was best when normalized to MYH (10.2–32.8%), with ACTN2 next (13.9–44.2%). The greater precision of normalization to MYH is due to less variability introduced by multiple laboratory steps with the Coomassie Blue staining of the post-transfer SDS-PAGE gel. Specifically, MYH measures the myofiber protein content of the biopsy sample loaded, without any western blot transfer or antibody detection. Detection of ACTN2 was done by western blot, introducing experimental variability with regard to electrotransfer and immunodetection, leading to higher variability in measures. The ACTN2 normalization led to lower dystrophin levels than other normalization approaches, possibly due to changes in ACTN2 content in the DMD biopsies used for standard curves.

The targeted quantitation of dystrophin using mass spectrometry is an orthogonal method to antibody-based assays like western blot and immunohistochemistry. The addition of stable isotope labeled muscle extracts to the normal muscle extracts prior to gel fractionation and in-gel digestion reduces sample to sample variation, thus increasing the robustness of the assay. In addition, the spiking of fully labeled muscle extracts ensures that other myofiber specific muscle proteins like filamin C can be used as internal controls for protein level normalization. The validated mass spectrometry method can be routinely used to detect low levels of dystrophin (LLOQ = 1%) in 50 µg total muscle protein extract.

The immunostaining protocol differed from published protocols in using a BMD patient biopsy in each experiment to set the gain of digital images for manual counting of dystrophin-positive myofibers, and digital ratios of overall fluorescence intensities of entire sections. We also used digital scans of entire slides with multiple sections, and quantitation of complete sections to avoid any bias in analyst selection of certain fields. This approach showed high reliability between analysts in quantitation of dystrophin-positive myofibers in measures of dystrophin-positive myofibers in a DMD biopsy with revertant myofibers, and in three female manifesting carriers (somatic mosaics for dystrophin expression).

The RT-PCR methods differed from other published protocols in using tissue cryosections as starting material. Our methods led to intact RNA of high molecular weight and excellent quality, and this in turn enabled single RT-PCR (30 cycles) with robust detection of dystrophin RT-PCR products of over 1.6 kb by multiple analysts.

RT-PCR methods described to date involve nested PCR (two rounds of PCR amplification), and this is considered a qualitative rather than a quantitative method [21,22].

Detection of drug-induced dystrophin protein in muscle biopsies from DMD patients is one of very few surrogate biomarkers acceptable for FDA accelerated approval pathways. Measures of dystrophin protein in patient muscle are technically challenging, given the large size (427 kDa) and low abundance in patient muscle. The orthogonal methods described here enable measures of drug-induced dystrophin protein and mRNA by multiple complementary assays, with both western blot and mass spectrometry showing good reliability and sensitivity; this will benefit future clinical studies and drug development programs in DMD.

Conclusion

Our study has successfully optimized four assays in an orthogonal approach to dystrophin protein and mRNA measures (western blot, mass spectrometry, immunostaining and RT-PCR) in support of a drug development program aimed to increase dystrophin expression in DMD patient muscle.

Executive summary

Background

- Increasing dystrophin in Duchenne muscular dystrophy (DMD) patient muscle can be a surrogate outcome for drug development in DMD.
- There is a need to have robust validated techniques that enable sensitive, specific and precise detection of dystrophin.
- The aim of this study was to describe validated techniques used for the detection of the biomarker, dystrophin, in muscle biopsies from DMD patients.

Materials & methods

- Four different assays were optimized in this study, western blot, mass spectrometry, immunostaining and RT-PCR, using a series of normal control, manifesting DMD carrier, Becker muscular dystrophy and DMD muscle biopsies.

Results

- The western blot results demonstrated that the specified acceptance criteria were successfully met, and that the technique is sensitive and precise with the ability to detect dystrophin at lower limit of quantitation of 1% normal levels.
- The mass spectrometry method used stable isotope labeled dystrophin as internal standard and filamin C for protein content normalization. Reproducibility, accuracy and sensitivity were confirmed in this study.
- For immunostaining, the methodology we introduced here is sensitive to detect the revertant muscle fibers and accurately quantitate the dystrophin protein level within the biopsy. In this technique, the Becker muscular dystrophy biopsy was used as a reference control tissue for the gain adjustment to avoid any bias during the analysis of the subsequent test samples.
- The reverse transcriptase PCR method used in this study is a single step (one-round) amplification method.

Conclusion

- The described orthogonal approach is appropriate to support clinical research aimed at increasing dystrophin in patient muscle.

Author contributions

K Uaesoontrachoon, K Mekhssian, H Montpetit, R Beauvois, A Keyhani, Y Hathout, T Yamashita, Y Satou, H Osaki, A Mullen, K Nagaraju and EP Hoffman were involved in the conceptualization, design and planning of experiments. K Uaesoontrachoon, S Srinivassane, J Warford, K Mekhssian, M Barkhouse, M Moraca, M Malbasic, W Ross, A MacKinnon, J Rowsell, M Matyas and S Mummidivapu were involved in performing the experiments. K Uaesoontrachoon was the principle author of the manuscript; while K Mekhssian, Y Hathout, T Yamashita, Y Satou, H Osaki and EP Hoffman provided editing support.

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K Uaesoontrachoon, S Srinivassane, J Warford, M Barkhouse, M Moraca, M Malbasic, W Ross, A MacKinnon, J Rowsell, A Mullen, M Matyas, K Nagaraju and E Hoffman are employees of AGADA BioSciences, a contract research organization. K Nagaraju and E Hoffman are co-founders and stock holders of AGADA BioSciences. Y Hathout is a paid consultant for AGADA BioSciences. K

Mekhssian, H Montpetit and A Keyhani are employees of Altasciences, a contract research organization. T Yamashita, Y Satou and H Osaki are employees of NS Pharma, a pharmaceutical company. Stock holders of NipponShinyaku Co. Ltd, parent company of NS Pharma. AGADA Biosciences and Altasciences are the contracted organization of NS Pharma Inc. as a funding sponsor, to perform this research for the development of validation methodology of detecting dystrophin protein. No resources including data and material related to clinical trials performed by NS Pharma Inc. have been utilized in this study. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. De-identified pre-existing pathological specimens (muscle biopsies) were used for the described study, with ethics approval by Veritas (Quebec).

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