Wrap-up: Measuring Dystrophin in Patients with Dystrophinopathies and Interpreting the Data

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- No conflicts of interest to report.
- The following represents what I think I have seen and heard today.

Measuring Dystrophin:

- Important now.
- Important for the future.
  - A sensitive and specific method to quantify dystrophin would be extremely useful for future drug development:
    - Selecting drug candidates
    - Selection of doses for study

Fundamental Issues in Quantifying Dystrophin

- Loss of dystrophin function is the cause of all dystrophinopathies.
- Dystrophin is present at a very low level in patients with DMD.
- Dystrophin levels in DMD patients who receive dystrophin restoration therapies are not normal, even if they are increased.
- Dystrophin is subject to degradation into fragments, which can confound quantitative techniques.

All Techniques: Variability is the 'Enemy' When Trying to Show a Treatment Difference

- between muscles/muscle groups
- within muscles – transverse and longitudinal
- storage and shipment conditions matter
- sample orientation
- disease progression
- tissue artifacts
- between staining runs; operators (technical factors)
- within a tissue section
- among fibers
- between operators (with respect to visual assessment)

Bias is Critical When Trying to Decide Whether a Detected Difference is Real

Bias is possible whenever a human makes a decision.

- Which patients to assess
- When to assess the patients
- What muscle group to assess
- Where in the muscle to sample
- How to handle the tissue sample
- Measurement technique, e.g., Ab, staining, field selection, and the analysis itself – numerous technical factors
- How to choose the endpoint
- How to deal with missing data
- How to analyze the data mathematically
- How to analyze the data statistically, interpret results

Muscle Sampling:

Muscle is one of the largest organ systems in the body

0.1 to 1 gram is a small sample!

Where should one sample?

- Depends on planned use.
- Select on basis of muscle function?
- Obtain a “representative” sample?
- Obtain the most “normal” sample?
- MRI-guided site selection?

How to Sample?

- Open biopsy
- Needle biopsy
- Fine needle biopsy

Open Biopsy:

Advantages

- success rate ~100%
- yield – plenty of tissue
- good when pathology is patchy (e.g., inflammatory myopathies)
- direct visualization (and ability to avoid) critical structures
- assurance of adequate hemostasis

Disadvantages

- invasive; inconvenient; causes apprehension
- post-op pain
- requires operating room; conscious sedation
- incision 1-4”; scarring
- cost $$

*** Open biopsy is less suitable for serial assessments.**

Needle Biopsy:

Advantages

- bedside procedure, done in clinic
- adequate for RNA or protein analyses
- local anesthesia for adults; conscious sedation for pediatric patients
- less invasive, less scarring
- amenable to serial sampling

Disadvantages

- loss of tissue architecture
- success variable, maybe 90-95% in experienced hands
- fewer accessible muscles
- local complications possible

*** Image-guided sampling can reduce complications**

Fine Needle Biopsy:

Advantages

- adequate for RNA/protein analysis
- bedside procedure, done in clinic
- least invasive
- generally well tolerated
- no scarring
- less costly
- most amenable to serial sampling

Disadvantages

- loss of tissue architecture
- success rate = ?
- produces smallest samples
- cannot be used for immunohistochemistry

Prior to Analysis: Factors That Can Influence Dystrophin Epitope Structure/Accessibility in Immunohistochemistry

- Fixation techniques
- Storage conditions
- Pre-treatment factors (rinsing; air-drying)

Must pay attention to such factors to avoid systematic error.

Desirable Qualities of a Monoclonal Ab

- Choice is critical for immunohistochemistry and Western blot.
- Need good specificity
  - cross-reaction with other proteins is a common problem, and can be specific to species and method
- Should work well across methods
  - immunofluorescence; Western blotting
- Many Abs are good; none are perfect

Antibody Binding Sites

- Antibodies have distinct binding sites.

Each antibody:

- recognizes a different protein epitope.
- has its own affinity.
- yields a different intensity value.

The binding domain and the affinity of the antibody are critical variables.

Immunohistochemistry

- Provides information on cellular localization.
- Works well to differentiate obviously positive cells from negative cells.
- Middle ground – 'grey area' – is the problem.
- Complex multistep procedure.
- Some question the linearity between fluorescence and protein level, especially at low protein concentrations, as in patients with DMD.

Revertant Fibers Can Complicate Interpretation of Immunohistochemistry

- Need to distinguish between revertant fibers and new dystrophin after exon skipping.
- Multiple types of revertant fibers can be present in a single biopsy.
- Detection depends on antibody used.
- Interpretation can be difficult.

Trace Fibers and Endogenous Fluorescence Can Complicate Interpretation

- Dystrophin traces: patchy, low levels of expression in many fibers throughout a biopsy.
- High variability.
- Essential to get high-quality biopsies, assess large fiber population; measure intensity along entire membrane.
- Try to reduce impact of background variability - important considering low intensity staining of DMD samples.

Immunohistochemistry – Reading the Slides

- Approaches range:
  - from having a single reader count positive fibers in randomly selected images from tissue sections, blinded to treatment group…
  - to elaborate systems using automated image analysis of every muscle fiber in a section.
- Select optimal magnification.
- ? Use confocal microscope.
- Some criticize blinded reading by an operator: human eye not particularly good at assessing subtle differences in intensity.

Immunohistochemistry – Technical Factors

- Detection of edges is subjective – can be done with image analysis.
- What is the area of interest? Should we compute average intensity per unit area (subjective because of edge detection)? Or intensity per unit length (i.e., perimeter of fiber)?
- Some suggest not scaling intensity to healthy controls, but instead scaling intensity in range appropriate for DMD samples.

Automated Analysis of Immunostaining
What is the Right Endpoint?

- Number of 'positive' fibers
  - Use total number of fibers as denominator (% positive)
  - Use muscle area as denominator (# positive/mm^2)
  - Use sample area as denominator (# positive/mm^2)
- Intensity of staining
  - Determine mean brightness of pixels above threshold
  - Count # of pixels above a particular threshold brightness

<u>Both methods need to be indexed to an area of interest</u>

- Per entire field? Per muscle fiber? Per length of cell membrane in microns?

Automated Analysis of Immunofluorescence

- can identify individual fibers
- can assess staining intensity of entire membrane of every fiber

BUT…

- Automated image analysis cannot overcome tissue artifacts or heterogeneous sampling.
- No reliable comparisons can be made between biopsies that differ in quality.
- Linearity between immunofluorescence intensity and dystrophin concentration cannot be established.
- Relative differences in immunofluorescence intensity between biopsies could be informative.

Western Blot

- Extremely powerful, standard laboratory technique.
- Semi-quantitative.
- Selection of antibody is critical.
- Complex method with many technical factors.
- Using a standardized protocol, concordance among 6 laboratories was good – better than with immunohistochemistry.

Western Blot – Technical Factors (1)

- 'Tug of war' between detection and saturation.
- There is a balance between lower limit of detection and standard curve range.
- Potential for degradation of dystrophin.
- Need baseline data, positive and negative controls.
- Need consistent sampling.
- The need to "overload" gel to visualize dystrophin can create distortions in migration, transfer and staining, especially of "loading control" protein.
- Saturation issues can lead to poor dynamic range.
- Need to determine lysates to be used to generate standard curve – DMD? Normal?

Western Blot – Technical Factors (2)

- Discussed some factors that can reduce distortions: use standard curve, spiking biopsies, avoid overloading
- Reference samples can be shared among laboratories for reproducible Western blotting.
- Normalization to total protein content could be considered.

Mass Spectroscopy

Advantages:

- Reproducible, replicate experiments over time.
- Linear over wide range (3 to 100% of normal)
- Limit of detection: can detect 0.1% of dystrophin relative to normal levels.
- Limit of quantification: can accurately measure as low as 3% of the amount of dystrophin relative to normal.

Disadvantages:

- Loss of spatial localization of dystrophin in muscle fibers
- Technically complex; requires state-of-the-art instrumentation and expertise
- Sample represents a tiny fraction of the patient.

Magnetic Resonance Imaging (MRI)

- Provides image of muscle composition.
- Can be used to direct biopsies
- Shows range of pathology throughout muscle group, replacement of muscle with connective tissue.
- Spectroscopy gives best quantification of fat fraction.
- Rate of progression of fat fraction - ? Study endpoint
- T_2 weighted imaging – images protons (water); sensitive to changes in membrane permeability, water content, edema, inflammation, fat content, fibrosis.

<u>Disadvantages</u>:

- Few centers have expertise.
- Boys have to be cooperative (i.e., remain motionless).
- Cost $$

Final

- Existing investigational therapies do not restore dystrophin to normal levels.
- Each method of quantification has strengths and weaknesses.
- We've a wealth of suggestions, from experts, on how to improve these techniques.
- Quantification is important now, and will be just as important in the future.

❖ **WE WOULD LIKE TO THANK EVERYONE FOR THEIR PARTICIPATION TODAY!**