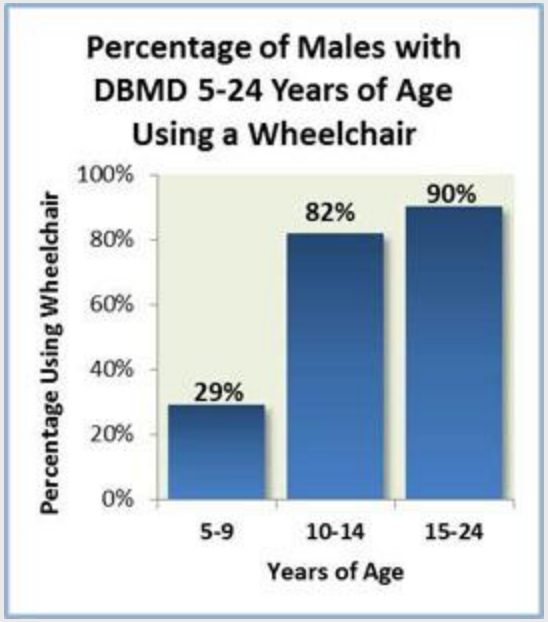
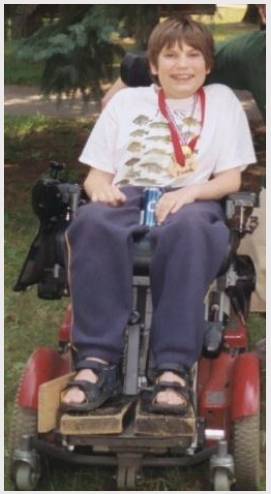
Duchenne Muscular Dystrophy

- Duchenne muscular dystrophy (DMD) is the most common form of muscular dystrophy, occurring in approximately 1 in 3,500.
- Promising new treatment to restore partially functional dystrophin protein by exon skipping is currently in clinical trials





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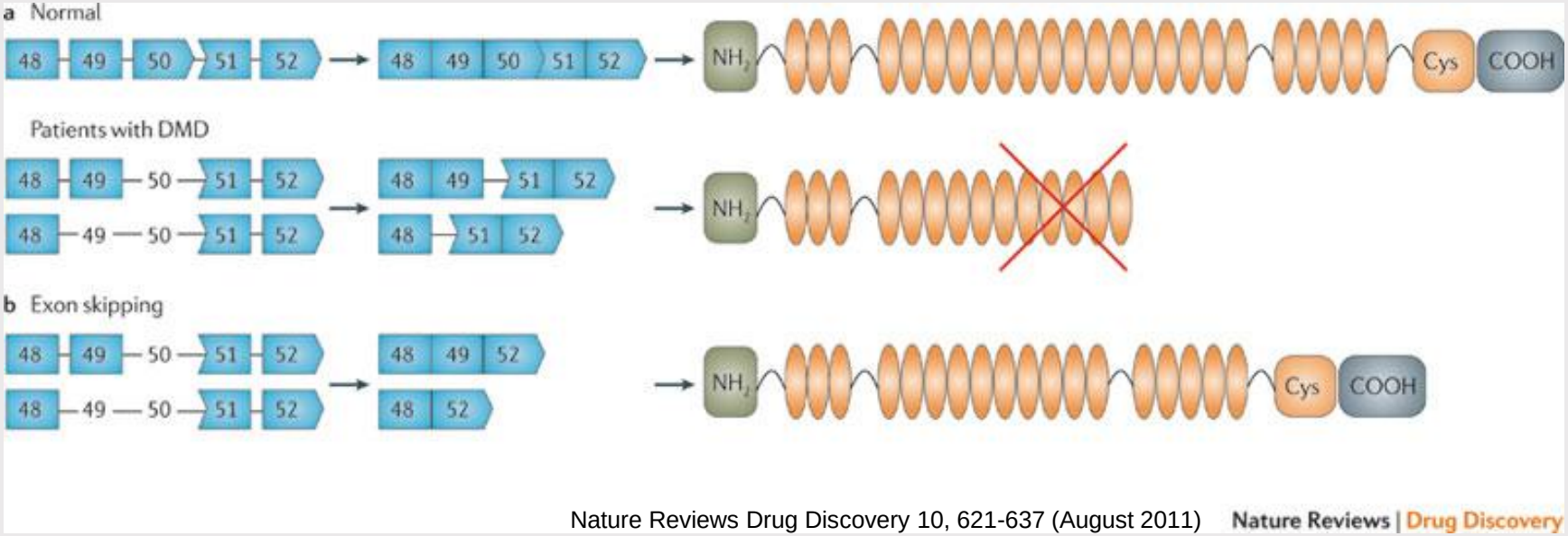
http://www.cdc.gov/ncbddd/musculardystrophy

Exon Skipping



Nature Reviews Drug Discovery 10, 621-637 (August 2011) Nature Reviews | Drug Discovery

Skip out of frame exons to put reading frame back in frame, resulting in a shorter Becker-like transcript

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Monitoring response to exon skipping therapy

- 6 minute walk--most widely used clinical endpoint
  - Subject to high variability
  - May take up to a year to show improvement
  - More patients required to power statistics
  - Not applicable for wheelchair bound patients

  } Longer trials and increased costs
- Skipped mRNA product quantitation
  - Indirect measurement
  - mRNA level do not always correlate to protein levels
- Dystrophin protein by immunohistochemistry or Western blot
  - Indirect measurement
  - Highly variable
  - Does not detect splice isoforms

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Dystrophin Quantitation

Goal is to use Dystrophin protein as an 'efficacy surrogate endpoint'.

- Dystrophin is a clinically relevant endpoint measurement
- Dystrophin is a low abundant protein, accounting for only 0.002% of total striated muscle
- Rapid analysis will aid critical Phase 2/3 assessments
- Need for a rapid, sensitive and reliable assay to quantitate dystrophin

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Dystrophin Quantitation

EMEA suggestion:
Convene international consensus working group to establish standards in dystrophin protein as endpoint

- Francesco Muntoni (London); Eric Hoffman (DC).
  - Call for participating testing labs
  - 7 labs volunteered
  - 1st Meeting to discuss approach
  - Distribution of biopsies, blinded
    - Test minimum of 3 replicates
    - Send data to central statistician
    - Intra-rater, inter-rater reliability defined
  - 2nd Meeting to discuss results

Immunoflourescence
Immunoblotting

- None reliable

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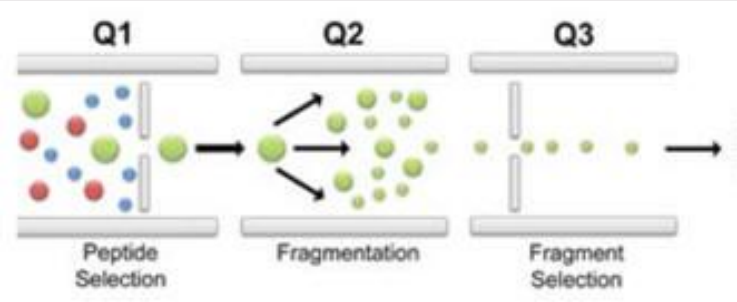
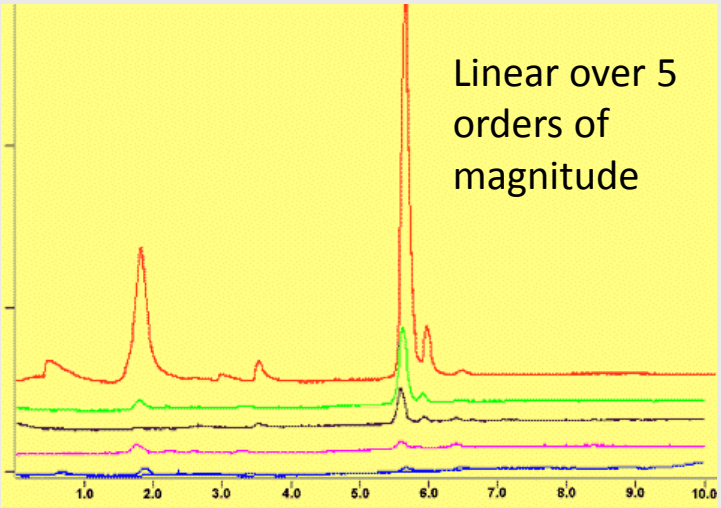
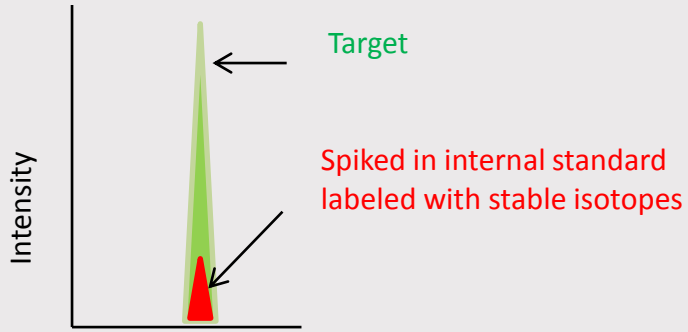
Need new assay:
MRM for targeted quantitative proteomics and validation



Multiple reaction monitoring is used to multiplex validation of selected targets in serial biological samples as an improved alternative to western-blot or ELISA. The FDA approve use as tool to measure surrogate biomarkers in a number of diseases.





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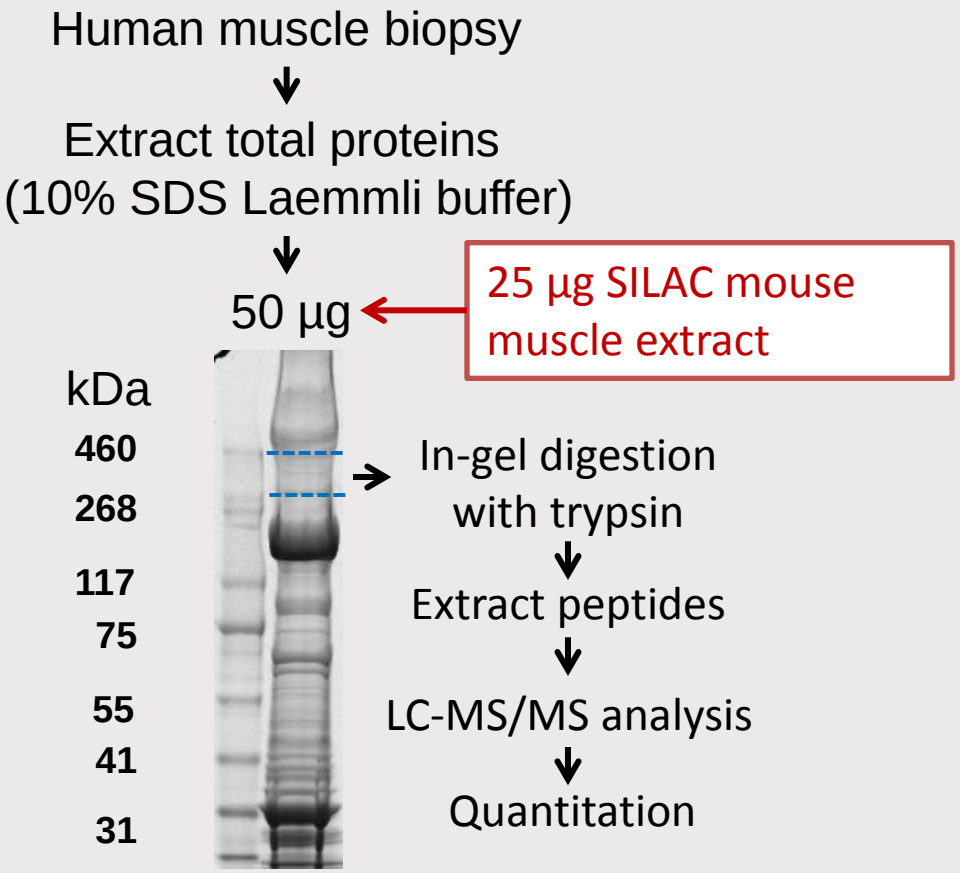
Targeted MS Quantification of Dystrophin and other proteins in Human Muscle Biopsies

- Mass Spectrometry based quantitation requires a stable isotope standard.
- We use labeled mouse muscle to serve as the labeled dystrophin.
- This “heavy” SILAC mouse was generated in-house by maintaining a colony on $^{13}C_6$-Lysine or ^{15}N feed.



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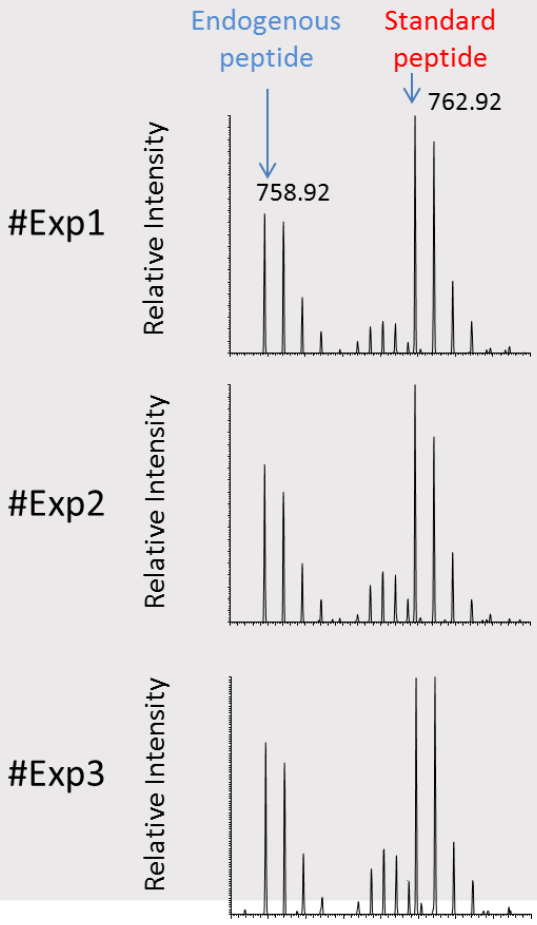
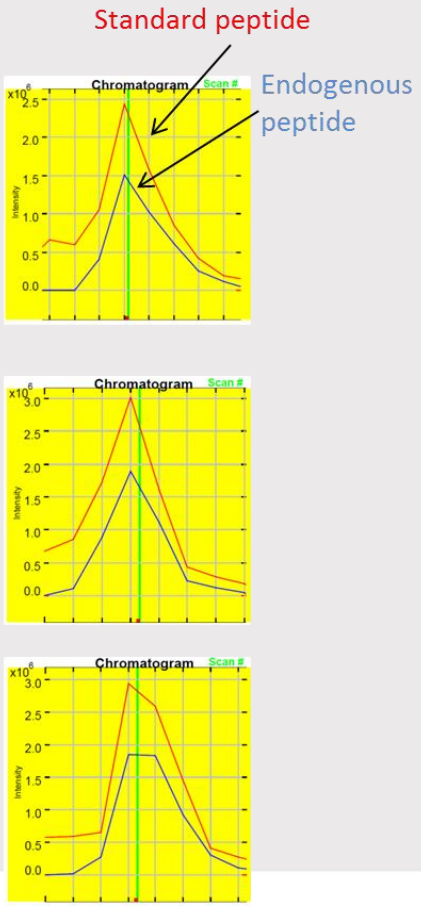
Accurate Quantitation of Dystrophin Protein in Human Skeletal Muscle Using Mass Spectrometry. Brown et al. J Bioanal Biomed 2013, S7

Reproducibility of the method performed on muscle samples from a healthy donor (< 7% CV)

MS

Extracted ion chromatogram





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Dystrophin Peptides Targeted by MS/MS

- >gi|5032303|ref|NP_004009.1| dystrophin Dp71ab isoform [Homo sapiens]
  - DAELIAEAK
    - 480.2558->418.2291
    - 480.2558->531.3131
    - 480.2558->644.3972
    - 480.2558->773.4398
  - DAELIAEAK[13C6]
    - 483.2659->424.2493
    - 483.2659->537.3333
    - 483.2659->650.4174
    - 483.2659->779.4600
  - LRQLLEQPQAEAK
    - 508.6228->418.2291
    - 508.6228->546.2877
    - 508.6228->643.3404
    - 508.6228->771.3990
    - 508.6228->507.2664
  - LRQLLEQPQAEAK[13C6]
    - 510.6295->424.2493
    - 510.6295->552.3079
    - 510.6295->649.3606
    - 510.6295->777.4192
    - 510.6295->510.2766
  - IFLTEQPLEGLEK
    - 758.9165->446.2604
    - 758.9165->785.4398
    - 758.9165->1042.5410
    - 758.9165->1143.5886
    - 758.9165->1256.6727
  - IFLTEQPLEGLEK[13C6]
    - 761.9266->452.2806
    - 761.9266->791.4600
    - 761.9266->1048.5612
    - 761.9266->1149.6089
    - 761.9266->1262.6929
  - QAPIGGDFPAVQK
    - 664.3538->542.3291
    - 664.3538->689.3975
    - 664.3538->918.4674
    - 664.3538->1031.5515
    - 664.3538->1128.6042
  - QAPIGGDFPAVQK[13C6]
    - 667.3639->548.3493
    - 667.3639->695.4177
    - 667.3639->924.4876
    - 667.3639->1037.5717
    - 667.3639->1134.6244
  - TLNATGEEIIQQSSK
    - 809.9177->577.2935
    - 809.9177->690.3775
    - 809.9177->932.5042
    - 809.9177->1118.5682
    - 809.9177->1219.6160
  - TLNATGEEIIQQSSK[13C6]
    - 812.9278->583.3137
    - 812.9278->696.3977
    - 812.9278->938.5244
    - 812.9278->1124.5885
    - 812.9278->1225.6361
  - VLSQIDVAQK
    - 550.8191->445.2764
    - 550.8191->560.3033
    - 550.8191->673.3874
    - 550.8191->801.4459
    - 550.8191->888.4780
  - VLSQIDVAQK[13C6]
    - 553.8292->451.2966
    - 553.8292->566.3235
    - 553.8292->679.4076
    - 553.8292->807.4661
    - 553.8292->894.4982
  - LLDLLEGLTGQKLPK
    - 546.6660->771.4717
    - 546.6660->941.5773
    - 546.6660->1070.6199
    - 546.6660->592.3556
    - 546.6660->706.4111
  - LLDLLEGLTGQK[13C6]LPK[13C6]
    - 550.6795->783.5121
    - 550.6795->953.6177
    - 550.6795->1082.6603
    - 550.6795->598.3758
    - 550.6795->712.4313
  - VHALNNVNK
    - 504.7828->474.2665
    - 504.7828->588.3094
    - 504.7828->701.3935
    - 504.7828->772.4306
  - VHALNNVNK[13C6]
    - 507.7929->480.2867
    - 507.7929->594.3297
    - 507.7929->707.4137
    - 507.7929->778.4508

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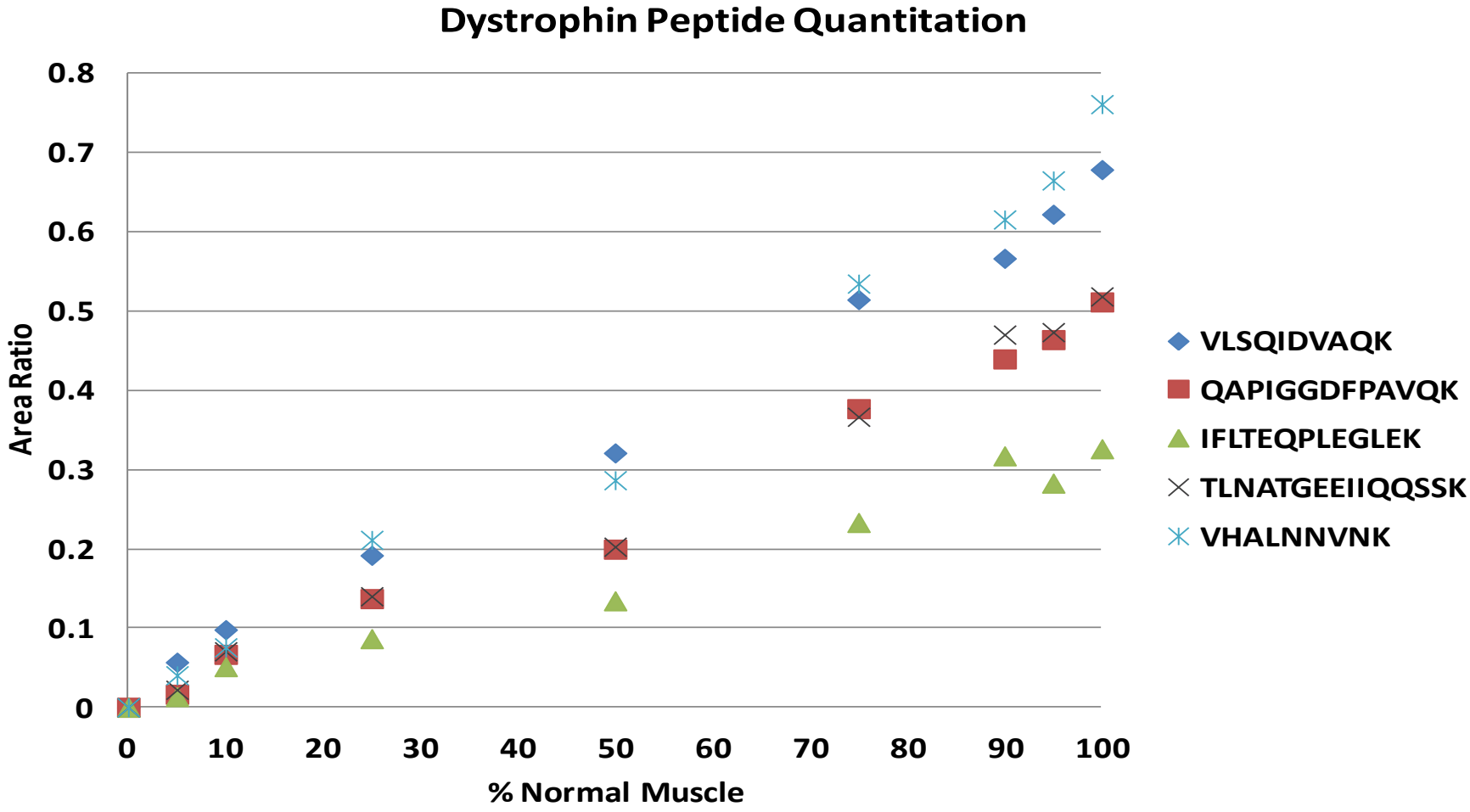
Dystrophin Peptide Quantitation w ith Increasing amount of Normal Muscle



Area response calculated by summing area across all gel slices.

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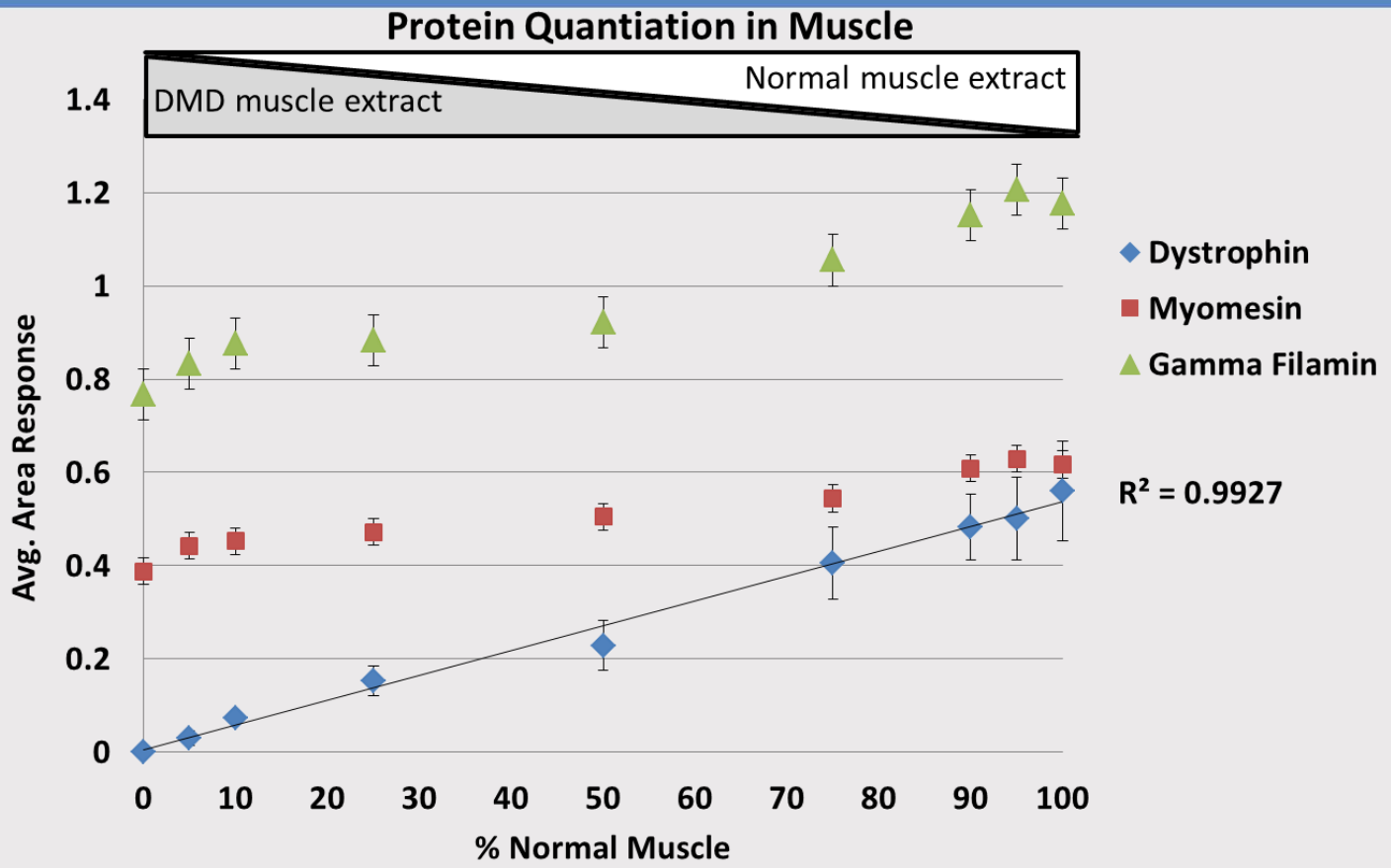
Peptide IFLTEQPLEGLEK dropped, 4 peptides used for final curve.

Quantification of Dystrophin Protein by Mass Spectrometry



- Linear response of DMD from 0 to 100%, detecting as low as 5% of Normal
- Muscle specific proteins serve as internal standards, with level response

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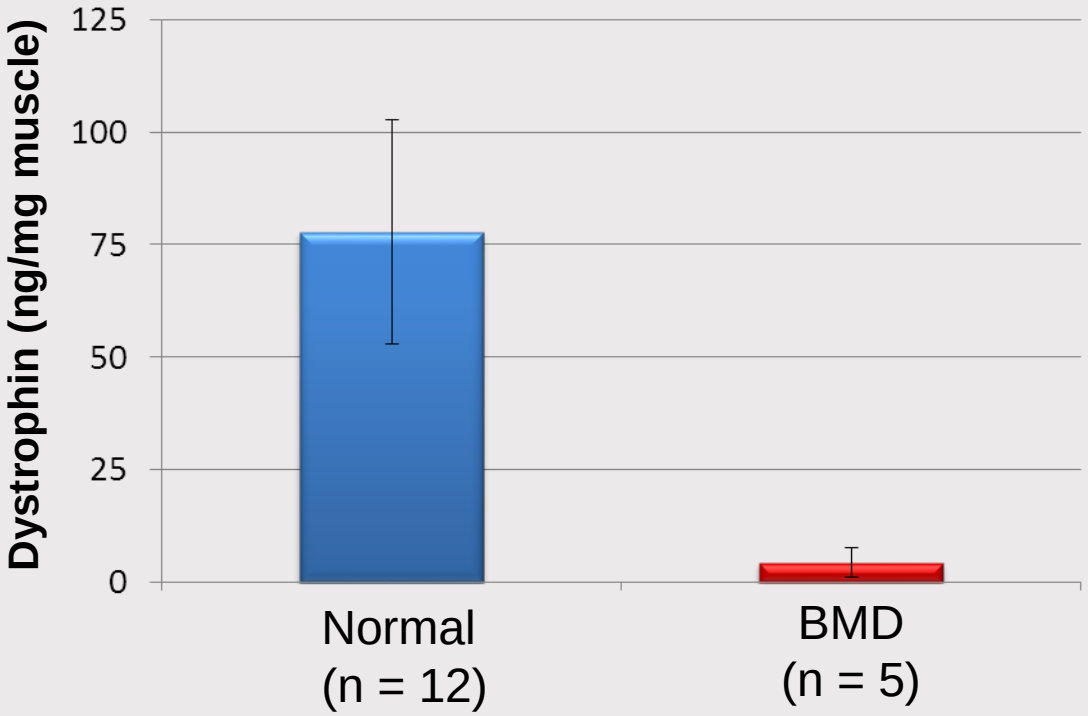
Brown et al. J Bioanal Biomed 2013, S7

Absolute Quantities of Dystrophin

Patient	Dystrophin (ng/50 ug extract)	Dystrophin (%)
normal 02	7.9	0.002
normal 06	3.8	0.001
normal 07	16.8	0.003
normal 08	18.4	0.004
normal 11	11.77	0.002
normal 14	5.12	0.001
normal 18	13.8	0.003
normal 25	nd	nd
normla 3071	5.3	0.001
normal 3154	nd	nd
normal 3233	3.07	0.0006
normal 3377	9.2	0.0018
Becker 3010	0.256	0.0001
Becker 3212	1.02	0.0002
Becker 3255	1.02	0.0002
Becker 3479	1.28	0.0003
Becker 7560	0	0.0000



* Values are normalized to filamin C

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FDA: Efficacy Surrogate Endpoint

Biomarker: “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to an intervention.” Example: cholesterol level.

Surrogate endpoint Biomarker: Biomarkers used for phase III clinical testing and biomarkers used to substantiate claims for product

“idea of a surrogate endpoint is to enable faster, smaller, more efficient clinical trials that can address urgent needs and facilitate the advancement of medicine”
Dr. Robert Temple of the Center for Drug Evaluation and Research (CDER)

http://www.nap.edu/catalog.php?record_id=12869

CDER Biomarker Qualification Program

Pre-Submission

1. Conference call (our current stage)
2. Cover Letter

Consultation & Advice

3. Letter of Intent
4. Qualification Review Team (QRT) Formed
5. Initial Briefing Package Submitted
6. Meeting with QRT
7. Briefing Documents submitted with revisions

Full Review

8. Qualification Package Submitted
9. Formal review by QRT and CDER
10. Final Decision

http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DrugDevelopmentToolsQualificationProgram/ucm284076.htm

Summary/Questions

- Dystrophin is a relevant surrogate biomarker for phase 2 trials aimed at restoring dystrophin expression.

- All dystrophin restoration therapies to date aim to produce abnormal dystrophin in patient muscle, not normal dystrophin

    - Stop codon read through: amino acid replacements

    - Exon skipping: internally deleted “Becker-like” dystrophin

    - Gene therapy: dystrophin mini-gene constructs

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Summary/Questions

- Abnormal dystrophin clearly therapeutic in animal models.

- Abnormal dystrophin clearly mitigates phenotype in Becker patients.

- Extensive genotype/phenotype studies suggest ~5%-10% needed:
  - Female skewed X inactivation patterns
  - Becker muscular dystrophy patients
  - Mouse models

- Is dystrophin testing of muscle a good surrogate biomarker to take through qualification process?

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