Modifiers:
What changes DMD

Kevin Flanigan, MD
Director, Center for Gene Therapy
Robert F. & Edgar T. Wolfe Foundation Endowed Chair In Neuromuscular Research
Nationwide Children’s Hospital
Columbus, Ohio
Disclosures

- Site principal investigator for PTC Therapeutics, Prosensa, Abeona Therapeutics, and the NIH FOR-DMD study; site co-investigator for Sarepta
- Advisory boards for Sarepta, PTC, Eli Lilly, 4D Therapeutics, and Dynacure
- Royalties from Audentes
Dystrophinopathies: Clinical diagnosis

DMD:
- Onset age 3-5
- Pelvic girdle weakness
- Tight heel cords
- CK 50-100X normal
- Loss of ambulation by age 12 (range 7-12)
- Mean age of death 19

BMD:
- Classic definition: loss of ambulation > age 12
- Alternatively:
  - “intermediate muscular dystrophy” for loss of ambulation ages 12 through 15
  - BMD for loss of ambulation > age 15
- Limb-girdle syndromes in adulthood
- Myalgias
- Isolated cardiomyopathy
Duchenne vs Becker: The “reading frame rule”

• Size of deletion does not correlate well with phenotype

• In-frame deletions are more likely to result in translation of a protein with partial function

• Out-of-frame deletions are DMD ~90% of the time

• What accounts for patients who break the reading frame rule?
• What accounts for variability among patients with DMD?
United Dystrophinopathy Project

Clinical evaluation by trained/validated evaluators + blood draw at clinical research sites (currently 7 centers)

DNA sample

Genetic testing at Utah Genome Center (Dr. Robert Weiss)

Historical and prospective data

UDP Database (NCH)

Online self-report registry with clinical + genetic data

Curation of genetic reports
UDP Genotype/Phenotype Database: enrollment diagnostic criteria

Clinical features of DMD/BMD and an X-linked family history
  Or

Muscle biopsy demonstrating absent or altered dystrophin expression (immunofluorescence, immunohistochemistry, immunoblot)
  Or

DMD gene mutation in previous clinical testing
Mutational Spectrum of DMD Mutations in Dystrophinopathy Patients: Application of Modern Diagnostic Techniques to a Large Cohort

Kevin M. Flanigan,1−4* Diane M. Dunn,1 Andrew von Niederhausern,1 Payam Soltanzadeh,1 Eduard Gappmaier,1 Michael T. Howard,1 Jacinda B. Sampson,7 Jerry R. Mendell,3 Cheryl Wall,3 Wendy M. King,5 Alan Pestronk,6,7 Julaine M. Florence,6 Anne M. Connolly,8 Katherine D. Mathews,8 Carrie M. Stephan,8 Karla S. Laubenthal,18 Brenda L. Wong,9,10 Paula J. Morehart,10 Amy Meyer,10 Richard S. Finkel,11,12 Carsten G. Bonnemann,11,12 Livija Medne,11 John W. Day,13 Jolene C. Dalton,13 Marcia K. Margolis,13 Veronica J. Hinton,14 the United Dystrophinopathy Project Consortium,1 and Robert B. Weiss1*  

1Departments of Human Genetics, University of Utah School of Medicine, Salt Lake City, Utah; 2Department of Neurology, University of Utah School of Medicine, Salt Lake City, Utah; 3Department of Pathology, University of Utah School of Medicine, Salt Lake City, Utah; 4Department of Pediatrics, University of Utah School of Medicine, Salt Lake City, Utah; 5The Research Institute of Nationwide Children’s Hospital and Ohio State University, Columbus, Ohio; 6Department of Neurology, Washington University at St. Louis, St. Louis, Missouri; 7Department of Pathology, Washington University at St. Louis, St. Louis, Missouri; 8Department of Pediatrics, University of Iowa, Iowa City, Iowa; 9Department of Pediatrics, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio; 10Department of Neurology, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio; 11Department of Neurology, The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania; 12Departments of Neurology and Pediatrics, The University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania; 13Department of Neurology, University of Minnesota, Minneapolis, Minnesota; 14Columbia–Presbyterian Hospital, New York, New York  

Communicated by Christophe Béroud  
Received 24 February 2009; accepted revised manuscript 5 August 2009.  
Published online 30 August 2009 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/humu.21114
# Reading Frame Rule in DMD/BMD

45%-55% of BMD patients have out-of-frame mutations.
Distribution of BMD versus DMD nonsense mutations

In-frame exons (39) shaded
Out-of-frame (40) unshaded

\[ p = 0.004 \]
Nonsense Mutations Do Not Always Predict DMD

- Mutations predicted as nonsense mutations may instead affect exon splice regulatory signals\(^1,2\)
  - This results in exclusion of exons
  - The remaining mRNA may be in-frame

---


©Disset A, et al. 2006. Published by Oxford University Press. All rights reserved.
Modifiers of phenotype:

• Modifiers at the DMD locus:
  • Altered mRNA splicing (nonsense-associated BMD)
  • Altered protein translation initiation
    o p.Trp3X allele (N. American founder allele)

• Other genes that modify disease severity
SPP1 genotype is a determinant of disease severity in Duchenne muscular dystrophy

- Cytokine involved in immune cell migration and survival
- Implicated in fibrosis through the TGF-beta pathway

**ABSTRACT**

**Objective:** Duchenne muscular dystrophy (DMD) is the most common single-gene lethal disorder. Substantial patient-patient variability in disease onset and progression and response to glucocorticoids is seen, suggesting genetic or environmental modifiers.

**Methods:** Two DMD cohorts were used as test and validation groups to define genetic modifiers: a Padova longitudinal cohort (n = 106) and the Cooperative International Neuromuscular Research Group (CINRG) cross-sectional natural history cohort (n = 156). Single nucleotide polymorphisms to be genotyped were selected from miRNA profiling in patients with severe vs mild DMD, and genome-wide association studies in metabolism and polymorphisms influencing muscle phenotypes in normal volunteers were studied.

**Results:** Effects on both disease progression and response to glucocorticoids were observed with polymorphism rs28357094 in the gene promoter of SPP1 (osteopontin). The G allele (dominant model; 35% of subjects) was associated with more rapid progression (Padova cohort log rank p = 0.003), and 12%-19% less grip strength (CINRG cohort p = 0.0003).

**Conclusions:** Osteopontin genotype is a genetic modifier of disease severity in Duchenne dystrophy. Inclusion of genotype data as a covariate or in inclusion criteria in DMD clinical trials would reduce intersubject variance, and increase sensitivity of the trials, particularly in older subjects.

*Neurology* 2011;76:219-226
Using mice to map genetic modifiers
Beth McNally, MD/PhD (Northwestern)

Parental strains: $Sgcn^{D2}$ (severe) $\times$ $Sgcn^{129}$ (mild)

F1 generation: $Sgcn^{D2/129}$

F2 generation $Sgcn^{D2/129}$

• 270 F2 animals were analyzed.
Membrane permeability and fibrosis are both modified by a region of chr 7.
An insertion/deletion in LTBP4 modifies muscular dystrophy

Heydemann et al. 2009
Gene expression

SECRETING CELL

TGFβ receptor

SMAD-P

LTBP

TGFβ

extracellular matrix

proteolysis

Large latent complex

LTBP + TGFβ

RECEIVING CELL

Gene expression
Does \textit{LTBP4} influence DMD phenotype?

- Use loss of ambulation as a dichotomous outcome

- To take an unbiased approach, we included all subjects who had a recorded loss of ambulation before age 20, regardless of what phenotype was recorded

- $N = 254$ subjects
  - 244 (96\%) were catalogued as DMD
  - 8 = IMD (lost ambulation between age 12 and 15)
  - 2 = BMD (lost ambulation after age 15 but before age 20)
LTBP4 Genotype Predicts Age of Ambulatory Loss in Duchenne Muscular Dystrophy

Kevin M. Flanigan, MD,1,2,3 Ermelinda Ceco, BS,4 Kay-Marie Lamar, BS,4
Yuuki Kaminoh, BS,1 Diane M. Dunn, BS,5 Jerry R. Mendell, MD,1,2,3
Wendy M. King, PT,3 Alan Pesronk, MD,6 Julaine M. Florence, DPT,6
Katherine D. Mathews, MD,7 Richard S. Finkel, MD,8 Kathryn J. Swoboda, MD,9
Eduard Gappmaier, PhD,10 Michael T. Howard, PhD,5 John W. Day, MD, PhD,11
Craig McDonald, MD,12 Elizabeth M. McNally, MD, PhD,4 and Robert B. Weiss, PhD5 for the United Dystrophinopathy Project

Objective: Duchenne muscular dystrophy (DMD) displays a clinical range that is not fully explained by the primary DMD mutations. LTBP4, encoding latent transforming growth factor-β binding protein 4, was previously discovered in a genome-wide scan as a modifier of murine muscular dystrophy. We sought to determine whether LTBP4 genotype influenced DMD severity in a large patient cohort.

Methods: We analyzed nonsynonymous single nucleotide polymorphisms (SNPs) from human LTBP4 in 254 nonambulatory subjects with known DMD mutations. These SNPs, V194I, T767A, T800A, and T1140M, form the VTTT and IAAM LTBP4 haplotypes.

Results: Individuals homozygous for the IAAM LTBP4 haplotype remained ambulatory significantly longer than those heterozygous or homozygous for the VTTT haplotype. Glucocorticoid-treated patients who were IAAM homozygotes lost ambulation at 12.5 ± 3.3 years compared to 10.7 ± 2.1 years for treated VTTT homozygotes or heterozygotes. IAAM fibroblasts exposed to transforming growth factor (TGF) β displayed reduced phospho-SMAD signaling compared to VTTT fibroblasts, consistent with LTBP4’s role as a regulator of TGFβ.

Interpretation: LTBP4 haplotype influences age at loss of ambulation, and should be considered in the management of DMD patients.

ANN NEUROL 2013;73:481–488
The \textit{LTBP4} “IAAM” haplotype predicts prolonged ambulation in DMD
Steroid use with *LTBP4* “IAAM” predicts prolonged ambulation in DMD
Haplotype analysis of nonsynonymous *LTBP4* variants associated with age of ambulatory loss

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>All (n=254)</th>
<th>Steroid treated (n=137)</th>
<th>Steroid naive (n=102)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Global P&lt;sup&gt;a&lt;/sup&gt; = 0.002</td>
<td>Global P&lt;sup&gt;a&lt;/sup&gt; = 0.013</td>
<td>Global P&lt;sup&gt;a&lt;/sup&gt; = 0.12</td>
</tr>
<tr>
<td>VTTT</td>
<td>freq 0.53</td>
<td>score -1.51</td>
<td>p-val&lt;sup&gt;c&lt;/sup&gt; 0.1</td>
</tr>
<tr>
<td></td>
<td>freq 0.52</td>
<td>score -1.04</td>
<td>p-val&lt;sup&gt;c&lt;/sup&gt; 0.3</td>
</tr>
<tr>
<td></td>
<td>freq 0.52</td>
<td>score -1.12</td>
<td>p-val&lt;sup&gt;c&lt;/sup&gt; 0.3</td>
</tr>
<tr>
<td>IAAM</td>
<td>freq 0.31</td>
<td>score 3.43</td>
<td>p-val&lt;sup&gt;c&lt;/sup&gt; 6 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>freq 0.32</td>
<td>score 2.92</td>
<td>p-val&lt;sup&gt;c&lt;/sup&gt; 0.004</td>
</tr>
<tr>
<td></td>
<td>freq 0.29</td>
<td>score 1.91</td>
<td>p-val&lt;sup&gt;c&lt;/sup&gt; 0.06</td>
</tr>
</tbody>
</table>

<sup>a</sup> The haplo.stats package was used to test for association between haplotypes and age of ambulatory loss as a quantitative trait using a recessive model.

<sup>b</sup> These haplotypes consist of SNPS: rs2303729, rs1131620, rs10880 respectively.

<sup>c</sup> p value from the \( \chi^2 \), df=1, distribution of the haplotype-specific score.

Mean age of ambulatory loss for “IAAM” versus other haplotypes:
- In steroid-treated patients: 12.5 ± 3.3 years vs. 10.7 ± 2.1 years
- In steroid-naïve patients: 11.2 ± 2.7 vs. 9.8 ± 2.0 years
LTBP4 “IAAM” fibroblasts have reduced TGFβ signaling
Our next step: Genome-wide association studies

• Instead of looking at a candidate gene (like $LTBP4$), what if we look at 2.5 million variants (SNPs, or single nucleotide polymorphisms) throughout the whole genome?

• Affymetrix OmniChip
• Same 254 subjects
Long-Range Genomic Regulators of THBS1 and LTBP4 Modify Disease Severity in Duchenne Muscular Dystrophy

Robert B. Weiss, PhD, Veronica J. Vieland, PhD, Diane M. Dunn, BS, Yuuki Kaminoh, BS, and Kevin M. Flanigan, MD
for the United Dystrophinopathy Project

Objective: Duchenne muscular dystrophy (DMD) is a severe X-linked recessive disease caused by loss-of-function dystrophin (DMD) mutations in boys, who typically suffer loss of ambulation by age 12. Previously, we reported that coding variants in latent transforming growth factor beta (TGFB)-binding protein 4 (LTBP4) were associated with reduced TGFB signaling and prolonged ambulation ($p = 1.0 \times 10^{-5}$) in DMD patients; this result was subsequently replicated by other groups. In this study, we evaluated whether additional DMD modifier genes are observed using whole-genome association in the original cohort.

Methods: We performed a genome-wide association study (GWAS) for single-nucleotide polymorphisms (SNPs) influencing loss of ambulation (LOA) in the same cohort of 253 DMD patients used to detect the candidate association with LTBP4 coding variants. Gene expression and chromatin interaction databases were used to fine-map association signals above the threshold for genome-wide significance.

Results: Despite the small sample size, two loci associated with prolonged ambulation met genome-wide significance and were tagged by rs2725797 (chr15, $p = 6.6 \times 10^{-5}$) and rs710160 (chr19, $p = 4.7 \times 10^{-6}$). Gene expression and chromatin interaction data indicated that the latter SNP tags regulatory variants of LTBP4, whereas the former SNP tags regulatory variants of thrombospondin-1 (THBS1): an activator of TGFB signaling by direct binding to LTBP4 and an inhibitor of proangiogenic nitric oxide signaling.

Interpretation: Together with previous evidence implicating LTBP4, the THBS1 modifier locus emphasizes the role that common regulatory variants in gene interaction networks can play in mitigating disease progression in muscular dystrophy.

ANN NEUROL 2018;84:234–245
SNPs found in a gene desert upstream from \textit{THBS1} locus
THBS1

• Encodes thrombospondin-1 (TSP-1)
• Multifunctional, extracellular matrix (ECM) glycoprotein that is a major activator of TGFβ signaling
  • Directly interacts with LTBP4 in the ECM.
• Also an anti-angiogenic factor
• THBS1 expression is elevated in mdx mice
(A) Two locus mean difference (years) in age at loss of ambulation

(B) 

- **THBS1, rs2725797**
  - decreased TSP-1

- **LTBP4, rs716160 / IAAM**
  - reduced latent TGFβ

Interaction:
- reduced CD47 receptor binding
- reduced TGFβ activation
- increased nitric oxide-cGMP signaling
- increased angiogenesis, blood flow
- reduced TGFβ signaling

Legend:
- ECM: Extracellular Matrix
- LTBP4: Latent TGFβ Binding Protein 4
Conclusions

• The TGFβ signaling pathway is a point of convergence for modifiers of disease severity.

• The *LTBP4* IAAM haplotype is associated with decreased TGFβ signaling.
  • seen independent of the primary mutation (truncating or not)
  • seen in both glucocorticoid treated and naïve DMD subjects

• This effect is amplified by the protective THBS1 allele.

• These modifiers should be considered in interpreting the results of clinical trials.
Ongoing search for new modifiers

• NIH (NINDS) project
• We are re-contacting families who participated in the United Dystrophinopathy Project to determine clinical outcomes, including
  • Age at wheelchair use
  • Age at diagnosis of cardiomyopathy
  • Age of BiPAP use
  • Survival
• Studying 2.5 million genetic locations (SNPs) within the genome for evidence of an association with better or worse prognosis.
• Exome sequencing in a subset of patients
• If you previously enrolled in the UDP, you may receive a call!
Acknowledgements

• University of Utah
  • Robert Weiss, PhD
  • Diane Dunn
  • Russ Butterfield, MD, PhD
  • Michael Howard, PhD
  • Kathy Swoboda, MD
  • Eduard Gappmaier, PhD
  • Jay Maiti

• University of Chicago
  • Elizabeth McNally, MD, PhD
  • Ermelinda Ceco
  • Kay-Marie Lamar

• Nationwide Children’s Hospital/OSU
  • Veronica Vieland, PhD
  • Megan Waldrop, MD
  • Tabatha Simmons, PhD
  • Roxane Alles, PhD
  • John Burian
  • Tori Danneker
  • Jerry Mendell, MD
  • Lindsay Alfano, DPT

• Washington University, St. Louis
  • Alan Pestronk, MD
  • Julaine Florence, DPT
  • Anne Connolly, MD

• University of Iowa
  • Katherine Mathews, MD

• Children’s Hospital of Philadelphia
  • Richard Finkel, MD

• University of Minnesota
  • John Day, MD, PhD

• University of California-Davis
  • Craig McDonald, MD