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TITLE: Modulating Calcium Signals to Boost AON Exon Skipping for DMD

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CONTRACTING ORGANIZATION:

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14. ABSTRACT

AON-mediated exon skipping is currently advancing as therapy for DMD, though levels of dystrophin produced remains suboptimal. Thus, identification of compounds with the capacity to boost exon skipping could help fully realize this potentially life-changing DMD treatment. We have assessed whether dantrolene, an already FDA-approved drug, can boost efficacy of AON exon skipping in the context of AON targeting skipping of exons 51, 44 or 45. Additionally, we have begun testing proprietary compounds that regulate the same Ca2+ pathway regulated by dantrolene for skip-boosting. .As a second objective we are assessing these compounds for their ability promote exon skipping in patient cells with DMD mutations that have a low level endogenous skipping, dystrophin expression and/or mild phenotypes. While we were unable to see consistant skip boosting in the absences of AON, we were able to demonstrate high level of endogenous skipping in DMD iDRM which are exon 44 skippable relative to other mutations. Natural history data demonstrate slower progression, likely as a result of this low level of dystrophin expressed, These data promise to inform clinical trial design as well as elucidate mechanisms or compensatory elements that predispose to DMD self correction/rescue..

15. SUBJECT TERMS

Exon skipping, Dantrolene, Calcium, Duchenne, Dytrophy, Dystrophin, anti-sense-oligonucleatide, DMD, RNA therapeutics.

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1. INTRODUCTION

AON-AON-mediated exon skipping is currently advancing as therapy for DMD, though levels of dystrophin produced remain suboptimal. Thus, identification of compounds with the capacity to boost AON-directed exon skipping may help fully realize this potentially life-changing treatment for DMD. Here, we will assess whether dantrolene, an FDA-approved drug already demonstrated to boost efficacy of AON exon skipping in the context of e45-50 DMD deletions, is also relevant to other mutations amenable to exon 51 skipping or to other exon 44, 45 or 53 AON/DMD mutation skip combinations currently in the clinical trial pipeline.
Additionally, we will test the two proprietary compounds that regulate the same Ca2+ pathway regulated by dantrolene for their activity in boosting AON-directed exon 51, 44, 45, or 53 skipping. Based on their known activity, these compounds promise greater efficacy and a wider therapeutic window than dantrolene. As a second objective we will assess the ability of these compounds to promote exon skipping in patient cells with *DMD* mutations that have a propensity for low level endogenous skipping, dystrophin expression and/or mild phenotypes. We hypothesize that these compounds may promote skipping in the absence of AON, and thus would represent a cost effective alternative to AON skipping for a subset of very rare mutations. Finally, we hypothesize that by combining chemical genomics with RNA Seq analysis we can begin to identify mechanisms of compound activity and specificity in order to guide second-generation drug discovery.

2. KEYWORDS

Exon skipping, dantrolene, Calcium, Duchenne, Dystrophy, dystrophin, anti-sense-oligonucleatide, DMD

3. ACCOMPLISHMENTS

Specific Aim 1: The major accomplishments of Specific Aim 1 are summarized in a manuscript we are preparing and results and discussion from a near final draft of this manuscript are included here, below. We hope to submit it for publication soon.

Tentative Title: Targeting RyR activity boosts antisense mediated exon 44 and 45 skipping in human DMD patient derived skeletal muscle and cardiac culture models

Florian Barthélémy, Richard, T Wang, Christopher Hsu, Emilie, D. Douine, S, F. Nelson, M.C. Miceli

Introduction

Duchenne Muscular Dystrophy is the most common lethal genetic disease of childhood, affecting 1/-5000 male births (Mathews et al., 2010; Romitti, 2015) and is caused by mutation of the *DMD* gene. *DMD* encodes the dystrophin protein, essential for the maintenance of skeletal muscle and heart health. Dystrophin is integral to the structure and function of dystrophin-associated glycoprotein complex (DGC), which provides structural support and membrane stability during muscle contraction by linking the actin cytoskeleton to the extracellular matrix (Tinsley et al., 1992). Additionally, dytsrophin controls asymmetric division in muscle stem cells (satellite cells), impacting stem cell renewaland myogenic fating during muscle repair. (Dumont et al., 2015). Without functional dystrophin, defects in sarcollemal membrane stabilization lead to progressive muscle damage, while satellite/stem cell defects limit effective regeneration. Progressive muscle weakness ultimately leads to respiratory and cardiac failure, and premature death between ages 20-30 (Bushby et al., 2010).

Most DMD mutations result from large frame-shifting deletions (~65%), (Bladen et al., 2015) leading to complete absence of dystrophin protein expression and Duchenne Muscular Dystrophy. A milder phenotype, referred to as Becker muscular dystrophy, often results from in-frame deletions which lead to the production of an internally truncated, but partially functional dystrophin protein. “Exon skipping” is a therapeutic strategy that uses antisense oligonucleotides (AO) to bypass one or more exons in the DMD pre- mRNA in order to restore reading frame and rescue expression of a partially functional dystrophin protein. Exon skipping AO have successfully restored mRNA reading frame and rescued dystrophin expression in human DMD and animal

DMD models (Mann et al., 2001; van Deutekom et al., 2001; Lu et al., 2005; McClorey et al., 2006a; McClorey et al., 2006b; Wilton et al., 2007; Yokota et al., 2009; Aoki et al., 2010). Initial human exon skipping studies focused on skipping exons 51, 53, 45, 44 and 8 since these rescue the most commonly occurring human DMD mutations and because mild phenotypes are associated with some in-frame deletions in these regions (Aartsma-Rus et al., 2005; Wilton et al., 2007; Aartsma-Rus et al., 2017).

Eteplirsen, a morpholino AON targeting Exon 51 skipping, recently received FDA accelerated approval based on the statistically significant induction of dystrophin expression as a surrogate bio-marker. (Mendell et al., 2016) (Aartsma-Rus and Krieg, 2017). Despite only low levels of dystrophin induction (<1%), treated patients showed delayed progression as measured by 6 MWD (six minute walk distance), loss of ambulation (LOA), and pulmonary function relative to external controls. It is anticipated that higher levels of dystrophin rescue will demonstrate even greater functional improvement. Thus, identification of agents capable of improving the efficacy of exon skipping hold potential to increase rescued dystrophin levels and functional benefit.

We previously published that dantrolene, an FDA-approved drug, boosts DMD AON mediated exon 51 skipping in human DMD fibroblasts, which were directly reprogrammed to myotubes through myoD induction (induced directly reprogrammed myotubes, termed iDRM). Further, systemic delivery of dantrolene boosted AO mediated exon 21 skipping and dystrophin protein rescue in mdx mice (Kendall et al., 2012) (Wang et al., 2018a). However, it remains unclear whether dantrolene is broadly applicable for boosting AOs reframing other DMD mutations and how it functions to promote skipping.

Dantrolene is known to target the RyR, which controls Ca+ regulated muscle contraction. Since we found that, like dantrolene, ryanodine and RyCalS107 also boost exon skipping, we hypothesized that Dantrolene targets the RyR to potentiate exon skipping.

Using DMD patient iDRM and iPSC derived myotubes to investigate exon 45 or exon 44 skipping, we demonstrate that dantrolene and RyCals S107 and ARM210 (a proprietary RyCal) can each boost AO directed skipping. Finally, in patient iPSC derived cardiomyocytes dantrolene is shown to boost AO exon 45 skipping These findings serve to further elucidate the applicability and mechanism of RyR modulators as boosters of AON exon skipping in human DMD.

Results

Dantrolene boosts DMD exon 45 and exon 44 skipping AO in patient-derived myotube cultures amenable to rescue by reframing. To determine if dantrolene AO skip boosting activity is applicable to promoting skipping of human DMD exons 44 or 45, we created cell models from patients eligible for exon 45 skipping (CDMD1003, DMDdelta46-51 and CDMD1006, DMDdelta 46-47) or exon 44 skipping (CDMD1015, DMDdelta 45)(Young et al., 2016; Wang et al., 2018b) and Figure 1. Comparative Genomic Hybridization confirmed the expected DMD mutations in primary fibroblasts (Figure 1A). RyR1 and Ryr2 expression were determined by RT-PC in DMD patient fibroblast directly reprogrammed to myotubes (iDRM) or first reprogrammed to induced pluurepoent stem cells (iPSC), and subsequently differentiated in culture to skeletal muscle myotubes or cardiomyocytes in culture (Figure 1B,C). Consistant with their known expression patterns, DMD iDRM and iPSC derived myotubes express RyR1, but not RyR2; whereas iPSC derived cardiomyocyte cultures predominantly express RyR2. The findings validate our reprogramming and differentiation schemes and demonstrate that RyRs, known dantrolene targets, are appropriately expressed in these model systems (Figure 1 B and C). Figure 1D provides a schematic indicating the DMD mutations present in each cell model used in our studies and the primers used for detecting mRNA lacking the skipped exon. (Figure 1D).

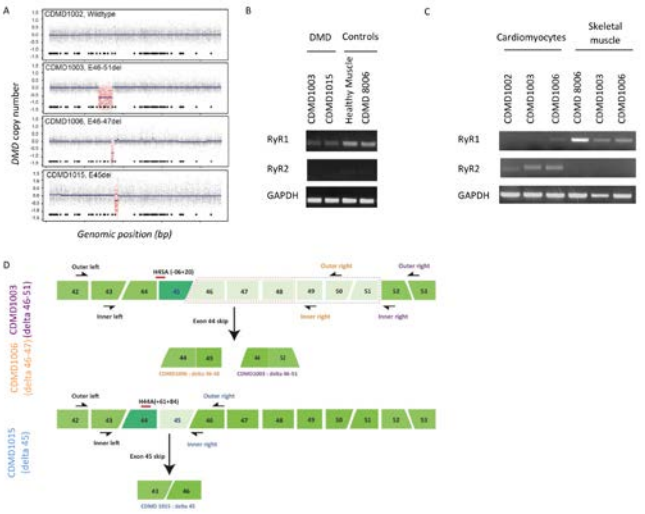

Figure 1 : Human **DMD patient and normal control cell based skeletal muscle and cardiomyocyte models used in this study.** A.***DMD*** **copy number variation confirms DMD mutations in primary DMD patient derived fibroblasts** . **A. custom CGH array was used to identify DMD gene mutations and breakpoint boundaries.** Displayed are copy number data from individuals with a wildtype *DMD* gene (1002) or DMD patients with deletion spanning exons 46-51 (CDMD 1003), 46-47 (CDMD 1015), or exon 45 (CDMD1006). Probes within the deleted *DMD* region are highlighted in red. Tiled below are the genomic locations of the 79 *DMD* exons, with exon 1 beginning on the right and ending with exon 79 on the left. Patient fibroblasts were reprogrammed to iDRM or iPSC and subsequently differentiated into skeletal or cardiac muscle lineage. B.**DMD iDRM derived myotubes express RyR1, but not RyR2.** Patient derived iDRM were differentiated to myotubes through induced myo-D activity and fusion conditions. Levels of RyR1 and Ryr2 expression were determined by RT-PC in DMD iDRM CDMD1003 and CDMD1015 derived myotubes relative to healthy control muscle biopsy or primary myoblasts (CDMD8006). C. **iPSC derived skeletal muscle predominantly express RyR1, whereas iPSC derived cardiomyocytes predominantly express RyR2 .** DMD iPSC CDMD1003 and CDMD1006 or healthy control iPSC CDMD 1002, 1006 , were differentiated to myotubes or cardiomyocytes and levels of RyR1 and RyR2 expression determined using RT-PCR. CDMD8006 are healthy primary myoblasts. D.**Exon skipping strategies. Schematic representing 3 patient mutations and strategies for exon skipping to restore reading frame.** Mutations for patients CDMD1003 and CDMD1006 are indicated by dashed boxes (purple and orange respectiviely). The location of primer pairs used for quantitating exon skipping are indicated outer primers for the first round of PCR and inner primers for the second round of PCR. Location and coordinates for AON H44A and H45A are also indicated.

First we tested the ability of dantrolene to boost exon 45 (iDRM CDMD1003, Fig. 2A) or 44 (iDRM 1015 Fig2A) skipping in iDRM derived cultured myotubes. As shown in figure 2, treatment with AO induces targeted exon (exon 45 or exon 44) exclusion and induction of skipped mRNA. Further, addition of dantrolene in combination with AO boosts the level of skipped mRNA relative to AO alone in both exon 44 and exon 45 skip amenable DMD cell models. (Figure 2).

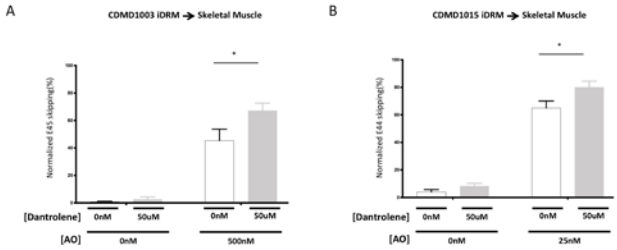

Figure 2 : Dantrolene boosts AON targeted exon45 and exon44 skipping in patient iDRM. iDRM CDMD1003 (delta 46-51) (A) or CDMD1015 (delta 45) (B) were differentiated to myotubes and treated with AON targeting exon 45 skipping, H45A, with or without dantrolene or dantrolene alone. Skipped and unskipped mRNA products were amplified using RT-PCR and quantified using Agilent 2100 Bioanalyzer. Data are expressed as normalized E45 or E44 skipping (skipped mRNA products over total mRNA products). (Bars represent SEM. *P<0.05 P values reflect a Students t test. Each point represents a single condition.

RyCals S107 and ARM210 boost AO directed exon 44 and 45 skipping. Next, we tested RyCals S107 and ARM210, for their ability to boost AO directed exon 45 or 44 skipping. We previously demonstrated that RyCal S107 can potentiate AO directed exon skipping in iDRM carrying a DMD mutation amenable to reframing by exon 51 skipping (Kendall et al., 2012). ARM210 is a novel RyCal currently under development as a potential stand-alone treatment targetting calcium defects in DMD hypothesized to be pathogenic. Here we demonstraten both CDMD1003 iDRM and iPSC derived myotube cultures, treatment with AO alone induces targeted exon 45 skipped mRNA (Figure 3).

Treatment with AO/dantrolene, AO/S107, or AO/ARM210 combinations each boost exon 45 skipping relative to AO alone.Similary, dantrolene and Rycals S107 and ARM210 also boost AO targeted exon 44 skipping in CDMD1015 iDRM patient derived myotubes (Figure 4).

To determine if the enhanced mRNA skipping in CDMD1015 was translated into enhanced dystrophin protein rescue we stained myotube cultures with antibody specific for dystrophin. After iDRM CDMD1015 differentiation, myotubes do not express dystrophin, consistant with the out of frame DMD mutation (Figure 4). Low levels of dystrophin staining can be detected in some myotube cultures treated with AO alone, and dystrophin levels are increased further when AO/dantrolene or AO/ARM210 are used in combination. These findings suggest that the observed increases in exon skipping translate into increased dystrophin protein rescue, consistant with our studies in mdx in vivo.

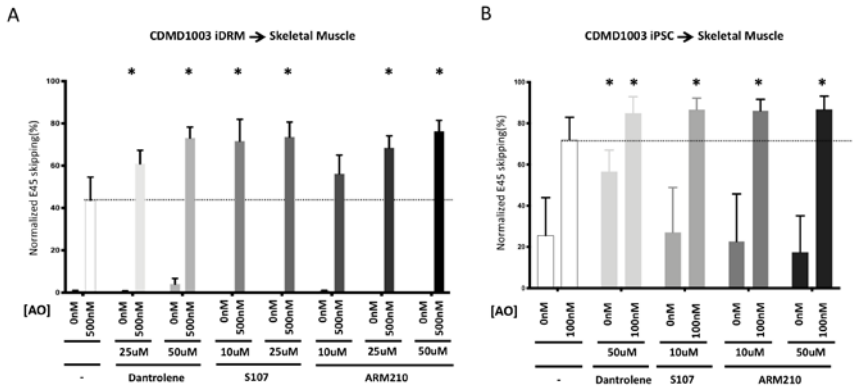

Figure 4 : Rycal ARM210 and Dantrolene boost AON exon44 skipping in patient iDRM derived myotubes to induce dystrophin protein. iDRM CDMD1015 (delta 45) were differentiated to myotubes and treated with AON targeting exon 44 skipping, H44A, with or without dantrolene or ARM210. Skipped and unskipped mRNA products were amplified using RT-PCR and quantified using Agilent 2100 Bioanalyzer (A). Data are expressed as normalized E45 or E44 skipping (mRNA skipped products over total mRNA products). (Bars represent SEM. *P<0.05 P values reflect a Students t test. Graphic represents two independent experiments run as triplicate. iDRM CDMD1015 (delta 45) were differentiated to myotubes and treated with AON targeting exon 44 skipping, H44A, with or without dantrolene or ARM210. Dystrophin protein was detected using ab15277 (C-ter) and Mandys 8 (rod domain) antibodies targeting dystrophin (B).

Discussion

While DMD exon skipping AOs have been well validated in animal models, only low levels of dystrophin rescue have been documented in human trials. While even these low levels likely slow disease progression, greater dystrophin induction is predicted to yield even better efficacy. Therefore, alternate chemistries, delivery mechanisms, or drug combinations that better promote exon skipping could be of significant value.

We previously identified dantrolene as a skip booster in a drug screen aimed at identifying compounds with the capacity to augment AO DMD exon skipping in a reporter line. Dantrolene AO skip-boosting activity has now been validated in mdx mice over both long- and short-term systemic AO treatment. In these studies, exon 23 AO/dantrolene combination therapy demonstrated higher levels of exon 23 skipped mRNA, rescued dystrophin protein and muscle function, and diminished pathophysiology relative to those treated with AO or dantrolene alone.

Dantrolene skip boosting activity was further validated in patient iDRM derived myotubes amenable to restoration of reading frame by exon 51 skipping, suggesting that boost activity may be relevant to human DMD exon skipping therapies. Both 2-0-methyl and morpholino AO were boosted by dantrolene, indicating that its activity may be agnostic to AO chemistry. However, we have yet to assess its activity in the context of other alternate chemistries or AO targeting other DMD mutations currently in the therapeutic pipeline.

Others compounds have been described with the ability to influence skipping in DMD models (6-thioguanine, NOL8, CELF2A antagonists)(O'Leary et al., 2009; Hu et al., 2010; Han et al., 2016; Martone et al., 2016). However dantrolene remains the most practical, since it is an FDA approved drug with a known safety profile, some previous exposure in DMD and the capacity to act in the context of human DMD mutations currently targeted by pipeline of approved AO skipping drugs.

Here we assess whether dantrolene is applicable to boosting AO skipping of exons 45 or 44, both of which are currently being targeted in preclinical or clinical studies. We demonstrate that dantrolene combines with exon

45 or exon 44 skipping AO to promote exon skipping in relevant DMD patient iPSC/iDRM derived myotubes. Similar results are seen across cell models derived from two different exon 45 skip amenable patients with distinct exon 45 mutations; DMDdelta46-51 and DMDdelta46-47. Likewise, dantrolene boosted exon 44 skipping in patient derived iDRM DMD myotube cultures carrying a mutation ammenable to reframing by exon 44 skipping (delta45). While the degree of boost is modest, it is in keeping with the levels boosted in mdx mice shown to impact pathology and in keeping with the literature indicating that even low levels of dystrophin can impact muscle function. Boost activity is seen in both patient iDRM and iPSC derived myotube models, adding confidence our findings. Of note the robustness of the differentiation into myotubes was more consistent in the iPSC and lower concentrations of AO (100nM) were required to induce AO or AO/dantrolene exon skipping relative to iDRM (500nM).

In skeletal muscle, Dantrolene targets RyR1; a calcium channel involved in the regulation of intracellular calcium release from the sarcoplasmic reticulum during muscle contraction. RyR1 signaling is impaired due to a calcium leakage in the muscles of DMD affected boys. For this reason, dantrolene has been testedin a small cohort of 8 boys over 2 years in an attempt to control the leak and slow DMD progression. Consistant with studies in the mdx mouse, trends toward modest reduction of serum CK and increased muscle function were observed, though the study was underpowered and did not reach statistical significance. Nonetheless, no adverse effects of dantrolene treatment were observed in this DMD cohort, aside from one patient in which dantrolene induced transient muscle weakness. This weakness was reversed upon lowering the dantrolene dose that the patient received for the duration of the study, highlighting the importance of monitoring potential acute weakness due to dantrolene treatment. While we saw no effect of dantrolene treatment alone on exon skipping or pathophysiology in mdx, it remains possible that in humans dantrolene may function alone to counter defective calcium regulation and downstream pathology in DMD as suggested by others findings, as well as a skip booster to reduce disease progression.

RyCals regulate RyR activity by stabilizing Calstabin:RyR binding and have been reported to normalize leaky RyR Ca+ flux observed in DMD muscle. For this reason, RyCal ARM210 has been considered as a stand-alone therapy for Duchenne. Site studies on Arm210 efficacy in mdx mouse and other human saftey studies. We previously demonstrated that RyCal S107 and ryanodine, like dantrolene, also boost exon 51AO skipping, leading us to hypothesize that the RyR is the relevant dantrolene target for promoting exon skipping. Here we demonstrate that RyCals ARM210 and S107, like dantrolene, both boost 45 or 44 AO targeted exon skipping, lending further support for skip regulation by RyR regulated pathway and identifying a novel RyCal with potential for boosting AO exon skipping in DMD. Because RyCals act to stabilize a closed conformation rather than block the active site, it has been suggested that they may not be limited by induced muscle weakness, which can be observed at high dantrolene doses in some patients. We have yet to determine the precise mechanism of RyR regulation of DMD exon skipping. However, previous studies have elucidate a role for tissue specific Ca regulation of alternative RNA splicing, providing a plausible mechanistic hypothesesis to test in future studies.

Specific Aim 2. Testing RyR pathway antags for activity on DMD patient with suspected propensity to skip.

We were unable to demonstrate consistant RyR antag boost in DMD cell models amenable to exon 44 or skipping in the absence of AON. However during the course of theses studies, we assessed a number of exon 44 skippable and other iDRM for skipped mRNA and were able to show that these mutations have a higher propensity to auto-skip. We published these findings together with a natural history assessment demonstrating that boys with these mutations progress less quickly. Our findings support the suggestion that the milder phenotype in individuals with 3-7 or 44 skippable results from low levels of dystrophin expression.
Our publication was highlighted as editors choice and our findings were featured on the cover. Wang, RT. Florian Barthelemy, Ann S. Martin[C], Emilie D. Douine, Ascia Eskin, Ann Lucas, Jenifer Lavigne Holly Peay, Lee Sweeney, Rita M. Cantor, M. Carrie Miceli[*] , Stanley F Nelson[*]. DMD Genotype Correlations from DuchenneConnect: endogenous exon skipping is a factor in prolonged ambulation for individuals

with a defined mutation sub-type. * Human Mutation. 2018, in press *Miceli and Nelson are joint senior authors.

What opportunities for training and professional development has the project provided?

These studies have served as a professional development opportunity for trainees Derek Wang, PhD candidate (anticipated graduation 2017) and postdoctoral fellow, Florian Barthelemy, PhD). In addition to providing them with greater technical proficiency working with human DMD models and exon skipping, presentation of initial findings at a poster at the New Directions in Muscle Cell biology meeting has provided presentation training to postdoctoral fellow Florian Barthelemy. Both trainees participate in the CDMD student and post-doctoral training program, which includes presenting and participating in biweekly CDMD inter-group meetings, an annual retreat, and hosting and attending seminars. While not a stated objective of this grant, trainee career development is a major focus of the CDMD.

How were the results disseminated to communities of interest?

Dr. Florian Barthelemy presented a poster reporting initial findings at the New Directions in Muscle Cell biology meeting, 2016.

Wang, RT. Florian Barthelemy, Ann S. Martin[C], Emilie D. Douine, Ascia Eskin, Ann Lucas, Jenifer Lavigne Holly Peay, Lee Sweeney, Rita M. Cantor, M. Carrie Miceli[*] , Stanley F Nelson[*]. DMD Genotype Correlations from DuchenneConnect: endogenous exon skipping is a factor in prolonged ambulation for individuals with a defined mutation sub-type. * Human Mutation. 2018, in press *Miceli and Nelson are joint senior authors.

4. IMPACT

Exon skipping is a now emerging therapy designed to correct the proximate genetic cause of DMD by inducing the expression of internally truncated protein associated with the much milder BMD. Once optimized it is predicted to significantly benefit up to 80% and enhance quality of life through slowing of disease progression. This will likely extend life directly. However, variation in patient response, suboptimal induction of dystrophin protein and clinical response clearly indicate that additional work is warranted to enhance the therapeutic potential of exon skipping. The work proposed here using RyCals or dantolene as adjuvant to exon skipping may make it more therapeutically beneficial or lead to the discovery of second generation boosters based on the pathway information gleaned.

Our recently published studies demonstrating that endogenous exon 44 skipping, especially enriched in DMDdelta45 patients, is correlated with milder disease progression will inform clinical trial design and lend insight into molecular strategies for DMD repair.

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

Our findings promise to provide the public knowledge regarding exon skipping and RNA therapeutics.

5. CHANGES/PROBLEMS

Nothing to Report

6. PRODUCTS

Publications, conference papers, and presentations

Dr. Florian Barthelemy presented a poster reporting initial findings at the New Directions in Muscle Cell biology meeting, 2018. See attached publication.

Wang, RT. Florian Barthelemy, Ann S. Martin[C], Emilie D. Douine, Ascia Eskin, Ann Lucas, Jenifer Lavigne Holly Peay, Lee Sweeney, Rita M. Cantor, M. Carrie Miceli[*] , Stanley F Nelson[*]. DMD Genotype Correlations from DuchenneConnect: endogenous exon skipping is a factor in prolonged ambulation for individuals with a defined mutation sub-type. * Human Mutation. 2018, in press *Miceli and Nelson are joint senior authors.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: M. Carrie Miceli
Project Role: PI
Researcher Identifier (e.g., ORCID ID): NA
Nearest person month worked: 2 cal mos
Contribution to Project: PI Dr. Miceli oversees all experiments helps with data interpretation and data publication.

Name: Stanley Nelson
Project Role: Co-I
Researcher Identifier (e.g., ORCID ID): NA
Nearest person month worked: 1 cal mos
Contribution to Project: Dr Nelson is an expert in RNAseq and DMD genotype phenotype correlations. He advises us on aims 1-2 and is key to the execution of Aim3.

Name: Florian Barthelemy
Project Role: Post Doctoral Fellow
Nearest person month worked: 6 cal mos
Contribution to Project: Florian Barthelemy: (post-doctoral fellow, Miceli lab) has taken the lead on developing all of the skipping reagents and performing assays assessing Rycal skip activity in a number of patient derived cells.

Name: Derek Wang
Project Role: Graduate Research Student
Nearest person month worked: 2 cal mos
Contribution to Project: Mr. Wang has assisted Dr. Barthelemy on assay development, ddPCR studies, patient cell banking reprogramming and differentiation.

Name: Ekaterina Mokhonova
Project Role: Staff Research Assistant
Nearest person month worked: 9 cal mos
Contribution to Project has been involved in banking cells and assessing pathway inhibitors in patient derived cells.

Name: Deirdre Scripture-Adams
Project Role: Assistant Researcher
Nearest person month worked: 6 cal mos
Contribution to Project: Helped with tissue banking and in trouble shooting reprogramming and differentiating patient fibroblasts to myotubes in culture.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Yes – please see Other Support page

What other organizations were involved as partners?

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

Nothing to Report

9. APPENDICES

Nothing to Report

OTHER SUPPORT

MICELI, M. CARRIE, Ph.D.

PREVIOUSLY ACTIVE GRANT HAS CLOSED

University of Florida, 8/01/15-7/30/18 0.6 cal months
PI: Lee Sweeney, site PI M. Spencer
Title: Failed Regeneration in the Muscular Dystrophies: Inflammation, Fibrosis and Fat
Major Goals: Examination of the immune response in the context of anti-fibrotic therapies.

CIRM TRX-05426 3/1/2013- 2/28/2015 3.6 cal months
PI: Stanley Nelson; Co-PI: M. Carrie Miceli
Title: Combination Therapy to Enhance AntiSense Mediated Exon Skipping for Duchene Muscular Dystrophy
Major Goals: To perform all preclinical assessment, dose ranging and efficacy and toxicology studies required to enable IND application and 100% clinical trial readiness for a combined therapeutic CDMD51Plus for use in exon skipping for DMD. Dr. Miceli will oversee all UCLA preclinical studies.

Penn/UCLA Wellstone Center Grant 7/01/10-6/30/15 0.6 cal months
PI: Lee Sweeney, University of Pennsylvania
Co-PIs/collaborators:Drs. McNally, Univ. of Chicago; Walter and Vandenborn; University of Florida; Spencer, Miceli, Nelson, UCLA and Ostap and Finkel, Penn
Title: Failed Regeneration in the Muscular Dystrophies: Inflammation, Fibrosis and Fat
Major Goals: As a collaborator with Dr. Spencer (subcontract PI), we examine the immune response to anti-fibrotic therapies.

UCLA Broad Stem Cell Research Center 8/25/14-8/24/15 1.2 cal months
Innovator Award
PI: M. Carrie Miceli
Major Goals: This award is to recognize and enable pursuit of Stem Cell Approaches to Combination Exon Skipping.

ACTIVE

P30 AR057230-06 4/1/14-3/31/19 1.2 cal months NIH/ NIAMS
PI: M. Spencer; Co-PI: M. C. Miceli
UCLA Muscular Dystrophy Core Center
(Miceli: Director of the High Throughput Screening and Cell Repository Core B and Member of the Executive Committee Core A). The goal of this grant is to establish a Muscular Dystrophy Center on the UCLA campus, consisting of research cores, pilot and feasibility funding and an administrative core that will facilitate translational research in the area of muscular dystrophy.

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RESEARCH ARTICLE

WILEY HGVS HUMAN GENOME VARIATION SOCIETY

DMD genotype correlations from the Duchenne Registry: Endogenous exon skipping is a factor in prolonged ambulation for individuals with a defined mutation subtype

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Abstract

Antisense oligonucleotide (AON)-mediated exon skipping is an emerging therapeutic for individuals with Duchenne muscular dystrophy (DMD). Skipping of exons adjacent to common exon deletions in *DMD* using AONs can produce in-frame transcripts and functional protein. Targeted skipping of *DMD* exons 8, 44, 45, 50, 51, 52, 53, and 55 is predicted to benefit 47% of affected individuals. We observed a correlation between mutation subgroups and age at loss of ambulation in the Duchenne Registry, a large database of phenotypic and genetic data for DMD ($N = 765$). Males amenable to exon 44 ($N = 74$) and exon 8 skipping ($N = 18$) showed prolonged ambulation compared to other exon skip groups and nonsense mutations ($P = 0.035$ and $P < 0.01$, respectively). In particular, exon 45 deletions were associated with prolonged age at loss of ambulation relative to the rest of the exon 44 skip amenable cohort and other *DMD* mutations. Exon 3–7 deletions also showed prolonged ambulation relative to all other exon 8 skippable mutations. Cultured myotubes from DMD patients with deletions of exons 3–7 or exon 45 showed higher endogenous skipping than other mutations, providing a potential biological rationale for our observations. These results highlight the utility of aggregating phenotypic and genotypic data for rare pediatric diseases to reveal progression differences, identify potentially confounding factors, and probe molecular mechanisms that may affect disease severity.

KEYWORDS
Duchenne muscular dystrophy, Duchenne Registry, rare disease registry

1 | INTRODUCTION

Duchenne muscular dystrophy (DMD, MIM # 310200) is a fatal X-linked disease characterized by a progressive loss of skeletal muscle function. It is most commonly caused by large deletions in *DMD* resulting in loss of dystrophin expression. With a prevalence of 1 in 3,500–5,000 live male births (Center for Disease Control and Prevention (CDC), 2009; Mathews et al., 2010), it is the most common

pediatric muscular dystrophy. Single or multiple exonic deletions and duplications account for 80% of mutations that cause DMD and the allelic disorder Becker muscular dystrophy (BMD; Bladen et al., 2015). Age at loss of ambulation (LOA) is variable in DMD, but typically, steroid-naïve boys lose independent walking ability between 9 and 11 years while those treated with corticosteroids ambulate on average for an additional 2–3 years (Angelini et al., 1994; Griggs et al., 1991, 1993; Henricson et al., 2013; Mendell et al., 1989; Wang et al., 2014). Steroid benefit has been clearly demonstrated in clinical trials, meta-analysis, and multiple natural history studies, and it has been the main drug intervention in DMD to date (Mendell et al., 1989).

Recently, AON-mediated exon-skipping strategies for DMD have shown promise in restoring myofiber expression of dystrophin and slowing disease progression (Goemans et al., 2011; van Deutekom et al., 2007). The dystrophin protein encoded by the 79 exons of the *DMD* transcript is among the largest in the proteome and consists of an N-terminal actin binding domain, 24 spectrin-like repeats, a cysteine-rich domain, and a C-terminal domain (Aartsma-Rus, Van Deutekom, Fokkema, Van Ommen, & Den Dunnen, 2006). Like DMD, the milder BMD is also often caused by large deletions within *DMD* but usually results in a preserved reading frame that results in expression of some functional protein (Monaco, Bertelson, Liechti-Gallati, Moser, & Kunkel, 1988). AON-induced exon skipping restores the reading frame in DMD by targeting specific exons for exclusion by the splicing machinery (Goemans et al., 2011; Koenig et al., 1989; van Deutekom et al., 2007). This has been shown to produce correctly localized functional dystrophin in mice (Mann et al., 2001), dogs (Yokota et al., 2009), and humans (Cirak et al., 2011). Targeting of exons 8, 44, 45, 50, 51, 52, 53, and 55 is predicted to help roughly 4%, 8%, 13%, 5%, 15%, 3%, 9%, or 2% of affected DMD boys, respectively (van Deutekom et al., 2001). However, the effect size for each of these AONs is not clear.

Aside from the "reading frame hypothesis," which demonstrated out-of-frame *DMD* exonic deletions typically caused DMD while in-frame deletions produced the milder BMD (Aartsma-Rus et al., 2006; Koenig et al., 1989), no concrete rules correlating frameshifting deletions in the mutational hotspot region and disease severity have emerged. However, exceptions to the reading frame rule exist: for instance, out-of-frame deletions of exons 3–7 sometimes result in a BMD phenotype, while in-frame deletions of exon 3 can exist in patients with a DMD phenotype (Kesari et al., 2008; Koenig et al., 1989; Tuffery-Giraud et al., 2009). Recently, some DMD individuals with mutations amenable to exon 44 skipping were observed to have a higher rate of revertant fibers and a larger number of trace dystrophin positive fibers relative to muscles from exon 51 amenable DMD boys (Lourbakos et al., 2011). Consistent with this description, several studies have indicated that exon 44 skip amenable DMD boys have an overall better functional outcome with higher age at LOA relative to typical DMD (Bello et al., 2016; Pane et al., 2014; van den Bergen, Ginjaar, Niks, Aartsma-Rus, & Verschuuren, 2014).

We sought evidence of correlation between age at LOA and genetic mutation subgroups in a large cohort of predominantly U.S. DMD patients using data available from the Duchenne Registry, a large patient-powered self-report registry for individuals or families affected by Duchenne and BMD (Rangel, Martin, & Peay, 2012; Wang et al., 2014). We data mined patient-reported parameters of age, ambulatory status, age at LOA, corticosteroid usage, and genetic mutation from 3,383 participants in the registry and corrected for the potential confounding effects of steroid usage. Patients with mutations correctable by skipping exon 8 or 44 ambulated significantly longer than deletion mutations correctable by skipping of exons 45, 50, 51, 52, 53, or 55, exon duplications, or nonsense deletions. The observed delay of age at LOA in boys with exon 44 targetable mutations was primarily due to patients with exon 45 deletion. Interestingly, boys with mutations correctable by skipping exon 51 lost ambulation earlier than the rest of the study cohort, and specific mutation types had different ages at LOA.

These clinical measures are supported by molecular evidence of endogenous exon skipping from cultured myotubes derived from patients at the Center for Duchenne Muscular Dystrophy (CDMD) at the University of California, Los Angeles. Significantly higher basal exon skipping was observed in myotubes derived from individuals with deletions of exon 45 or exons 3–7 compared to those with deletions of exons 45–50 or 49–50, indicating a potential explanatory cause for the biostatistical results.

2 | MATERIALS AND METHODS

2.1 | Data collection

The Duchenne Registry is an online self-report registry (www.duchenneregistry.org) for individuals and families affected by Duchenne and BMD (Rangel et al., 2012). Participants respond to questionnaires for specific topics: diagnosis, muscle function, corticosteroid, cardiac, respiratory, family history, and genetic testing. Genetic testing reports are reviewed by certified genetic counselors and annotated in a standard format. We downloaded the deidentified October 2016 freeze of the Duchenne Registry dataset which contains responses from 108 countries. Preliminary quality control and filtering were performed as previously described (Wang et al., 2014). Briefly, we filtered the data for males who reported valid and consistent ambulation status, corticosteroid usage/nonusage, and genetic mutation results confirmed by submission of genetic report to the Duchenne Registry genetic counselors. Participants were limited to residents of member nations of the Organization for Economic Cooperation and Development to ensure comparable levels of health care quality and access. For this analysis, genetic testing, corticosteroid, and muscle function modules were assembled per individual resulting in 1,913 complete profiles (Figure 1). Patients were then stratified into separate groups according to either the predicted exon skip necessary to produce an in-frame transcript (exon 8, 44, 45, 50, 51, 52, 53, or 55), possession of frameshifting exonic duplication or nonsense mutation or other exonic deletions not currently targetable by exon skipping.

All participants of the Duchenne Registry consented during registration for their deidentified data to be shared with researchers. Downloaded, deidentified data were deemed exempt by University of California Los Angeles Institutional Review Board.

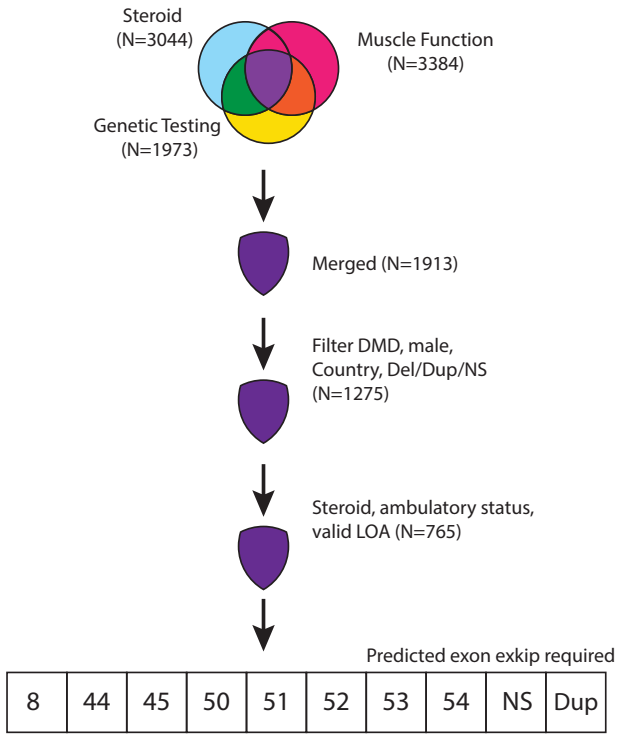

FIGURE 1 Filtering steps of Duchenne Registry data for analysis in this study. Participants can respond to one or more survey modules on steroid use, genetic testing results, or muscle function. Data for individuals who responded to all three submodules were merged. We removed entries that did not include valid diagnosis, mutation type, country of residence, steroid usage, ambulatory status, and age at LOA status. Individuals amenable to exon skipping were then sorted into the predicted exon skip required to generate in-frame *DMD* transcript

2.2 | Statistical analysis

Kaplan–Meier analysis was used to assess differences in age at LOA among the Duchenne Registry respondents when grouped by varying genetic mutations. Individuals considered in our analysis were currently using corticosteroids, possessed a defined mutation and a valid age at LOA. Age at LOA was defined as the age at which a boy required full-time wheelchair use as entered in the Duchenne Registry database. Ambulatory participants were included as right-censored data to augment statistical power. Age at LOA has been used by several other studies because it is a significant milestone and is well recalled by patients or their families. Log-rank test was used to statistically test for differences between Kaplan–Meier plots of age at LOA among genetic mutation groupings. Cox proportional hazards regression was used to estimate the effect of individual variables on age at LOA. Differences due to mutation subgroups were quantified as hazard ratios (HR), with confidence intervals (CI). HR < 1 indicated delayed age at LOA. We included the same mutational subgroups above regardless of corticosteroid status while using age at LOA as outcome variable. Statistical analysis was performed using R version 3.3.1 (64 bit) and version 2.37 of the Survival package. Kaplan–Meier analysis on steroid naïve patients was not attempted due small sample sizes.

2.3 | Collection, isolation, and propagation of dermal fibroblasts and myoblasts

Skin punches were obtained with informed consent from patients of the CDMD under University of California Los Angeles IRB-approved protocol (#11-001087). Isolation of dermal fibroblasts followed published protocols (Karumbayaram et al., 2012). Cells were reprogrammed using a tamoxifen-inducible MyoD overexpression system as previously described (Kendall et al., 2012) to create induced directly reprogrammed myotubes (iDRM). Primary myoblasts were purified from 50–100 mg of tissue obtained by needle muscle biopsy of vastus lateralis. Each core biopsy was dissociated into 1-mm pieces in a 1:1 MIX of dispase (1.5 U/mL)/collagenase (1,000 U/mL). After 20 minutes at 37°C, chunks were triturated and passed through a 70-μm cell strainer. Cell suspension was centrifuged at 1,200 rpm for 4 minutes, and the pellet was resuspended in 10 mL growth media (Nutrient Mixture F-10 HAM with 20% FBS and 1% pen/strep) and plated into a T75 flask. After 1 hour of preplating, the cell suspension was placed into a new T75 flask, which was considered to be enriched in myoblasts. All derived cells are assigned a patient unique study ID to allow inclusion of clinical data including mutation type and clinical progression.

2.4 | In-culture myotube formation

Inducible directly reprogrammable myotubes (iDRMs) were seeded at 200,000 cells per well in fibroblast growth media (DMEM (+phenol red, high glucose) + 15% FBS + 1% nonessential amino acids + 1% pen/strep) in 6-well plates (Corning) precoated for 1 hour with 0.1% gelatin (Sigma). The following day, 5 μM 4OH-tamoxifen (Sigma; resuspended in ethanol) was added in fibroblast growth media for 24 hours. On day 3, cells were washed in 1× phosphate-buffered saline (PBS; Invitrogen), and fusion media containing 1 μM 4OH-tamoxifen was added (1:1 Ham's F-10:DMEM (phenol red free, high glucose), 2% horse serum, 2% insulin–transferrin–selenium). On day 7, cell pellets were harvested and frozen for subsequent RNA isolation and endogenous exon-skipping analysis via nested PCR with primers encompassing the deleted region.

Primary myoblasts were cultivated in growth media as described above but supplemented with 5 ng/mL of bFGF starting at day 3. The media were changed every 3 days until confluence was reached. At confluence, media were exchanged for skeletal muscle differentiation media (Promocell) for 7 days before being harvested in TRIzol for RNA isolation (Ambion).

2.5 | RNA isolation, PCR, and quantification

Total RNA was isolated using the Purelink RNA mini kit (Ambion). For exon-skipping analysis, 200–500 ng of total RNA was reverse transcribed with random hexamers (Life Technologies). For cell lines with a deletion of exon 45, nested PCR was performed between exons 42–46 (Ex42-o: CAATGCTCCTGACCTCTGTGC + Ex 46-o: GCTCTTTTCCAGGTTCAAGTGG and Ex 43-i: GTCTACAACAAAGCTCAGGTCG + Ex 46-i: GCAATGTTATCTGCTTCCTCCAACC). For cell lines with a deletion of exons 45–50, we used primers previously described (Kendall et al., 2012). For cell lines with a deletion of

exons 49–50 or 48–50, a nested PCR was performed between exons 42 and 46 (Ex47-o: AGGACCCGTGCTTGTAAGTG + Ex 55-o: TCTTCCAAAGCAGCCTCTCG and Ex 47-i: AGCAGACAAATCTCCAGTGGA + Ex 53-i: TTCAACTGTTGCCTCCGGTT). After the verification on agarose gel, samples are loaded on a DNA1000 Bioanalyzer chip and run on the 2100 Bioanalyzer (Agilent Technologies). This technology plots fluorescence intensity versus migration time and produces an electropherogram for each sample. Results are analyzed by quantitating the molarity of products within the peaks at the expected sizes. Percentage of exon skipping was calculated as the molar amounts of $\frac{\text{skipped PCR product}}{\text{skipped PCR product} + \text{unskipped PCR product}} \times 100$. For cell lines with a deletion of exons 3–7, primers were used as previously described (Fletcher et al., 2012).

2.6 | Immunofluorescence on cultured myotubes

Primary myoblasts were grown as described above. After induction of differentiation for 7 days, cells were fixed with acetone for 7 minutes at −20°C. Dystrophin was detected using MANDYS8 (directed against the central rod domain, sc-58754, Santa Cruz Biotechnology) at a dilution of 1:100. Secondary goat anti-mouse (A32731, Life Technologies) was used at a dilution of 1:500. Images were obtained using a Zeiss microscope at a 20× magnification and processed with Axiovision software and/or ImageJ software.

2.7 | Muscle biopsy immunohistochemistry

Muscle biopsies were flash frozen in liquid nitrogen–cooled isopentane and stored at −80°C. At the time of processing, biopsies were mounted in OCT (Tissue-Tek) and 10-μm transverse cryosections were obtained. These were treated in 3% aqueous solution of hydrogen peroxide for 10 minutes and were blocked with 2.5% normal horse serum (Vector S2012) for 30 min at room temperature. Sections were incubated in primary antibody at 37°C degrees for 1.5 hours (dystrophin rod domain: NCL-DYS1, 1:50, Leica Biosystems; dystrophin C-terminal NCL-DYS2, 1:50, Leica Biosystems) and (dystrophin 3: NCL-DYS3, 1:50, Leica Biosystems; antibody diluent: DAKO S3022). Subsequently, the slides were rinsed in PBS and placed in secondary antibody for 1 hour. The sections were developed using DAB reagent (DAB substrate ki9t, vector SK-400), counterstained in hematoxylin for 2 minutes, dehydrated in graded alcohol and xylene, and coverslipped with Permount. Images were obtained as for cultured myotubes.

3 | RESULTS

Kaplan–Meier analysis was performed on males with DMD who were currently using corticosteroids and had deletion mutations amenable to targeted skipping of exons 8, 44, 45, 50, 51, 52, 53, or 55, as well as exonic duplications or nonsense mutations. Age at LOA, defined as the age at which a male with DMD required full-time wheelchair use, was the outcome variable.

Three groups showed statistically significant differences in age at LOA compared to the remainder of the group by a log-rank test (Figure 2). Individuals potentially amenable to exon 44 skipping

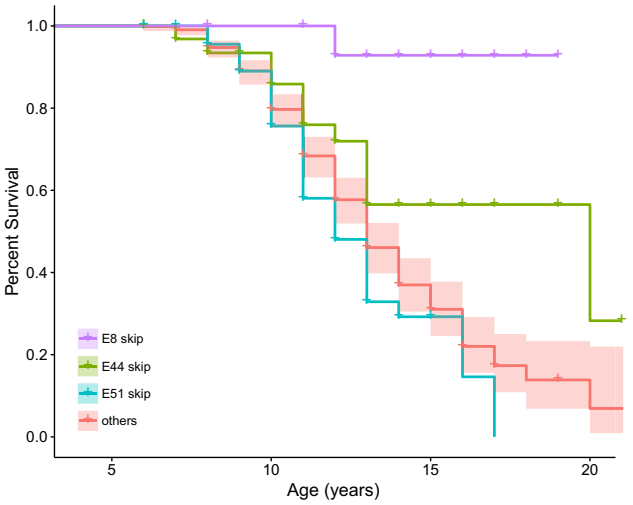

FIGURE 2 Kaplan–Meier age at LOA analysis for patients eligible for skipping therapy of exons. Delayed age at LOA was observed among individuals amenable to exon 8 skipping ($P < 0.001$) and exon 44 skipping ($P = 0.04$). Exon 51 skippable individuals had earlier age at LOA ($P = 0.04$). All other groups (45, 50, 52, 53, duplication and nonsense) were not significantly different and were merged. All subjects were currently using corticosteroids

therapy (e44 skip; $N = 74$) showed a difference in age at LOA by the log-rank test ($P = 0.035$), with a striking median age at LOA of 20 years, in contrast to the median age at LOA of 13 for the remainder of the cohort. The large majority of boys amenable to exon 8 targeted therapy ($N = 18$) were still ambulatory at age 20 ($P < 10^{-5}$), and most of these boys have exon 3–7 deletion, which is often affiliated with a BMD phenotype (Muntoni et al., 1994). Consistent with previously reported cohort studies of DMD, the largest group consisted of individuals amenable to exon 51 therapy (e51 skip; $N = 106$). This group has a more severe disease progression with an observed earlier median age at LOA at age 12 ($P = 0.04$). No other DMD mutation subgroups showed significant differences in age at LOA (Table 1, Supporting Information Figure S1).

Among mutations amenable to exon 44 skip therapy, 64% were due to a single mutation type: exon 45 deletion. Age at LOA in individuals with single exon 45 deletions ($N = 49$) was delayed compared to the other exon 44 skippable mutations and the remainder of the Duchenne Registry population ($P = 0.029$; Figure 3). Thus, these data indicate that the prior observations of exon 44 skippable patients having a milder phenotype may be restricted to the exon 45 deletion subgroup. Exon 3–7 deletions, amenable to exon 8 skipping therapy, were also able to ambulate significantly longer than other groups ($P = 0.0003$). For reasons that are not entirely clear, we also observed that the subgroup of exon 49–50 deletion DMD subjects ($N = 24$) that are amenable to targeted exon 51 skipping therapy were more likely to lose ambulation earlier than all other mutation groups ($P = 0.008$; Figure 3) with a median age at LOA of 11.

We applied Cox regression analysis on 961 individuals to estimate the HR of corticosteroid usage and mutation subgroup on the age at LOA. Exon 44 skippable mutations and exon 8 skippable mutations (e8 skip) were statistically significant explanatory variables for age at LOA

TABLE 1 Table of mutation subgroups and log-rank test *P* values

Mutation subgroup	*N*	%	Median age at LOA (years)	Log-rank *P*
Exon 8 skippable	18	2.4	NA	**<0.01**
Exon 44 skippable	74	9.7	20	**0.04**
Exon 45 skippable	70	9.1	13	0.80
Exon 50 skippable	33	4.3	16	0.24
Exon 51 skippable	106	13.8	12	**0.04**
Exon 52 skippable	29	3.8	16	0.52
Exon 53 skippable	78	10.2	12	0.62
Exon 55 skippable	21	2.7	13	0.24
Duplication	83	10.8	13	0.50
Nonsense	71	9.3	14	0.59
All other exonic deletions	182	23.8	13	NA

Log-rank *P* value is for comparison between specific subgroup compared to all other subgroups in aggregate. Significant tests with $P < 0.05$ are bolded. *N* denotes number of individuals. LOA, loss of ambulation.

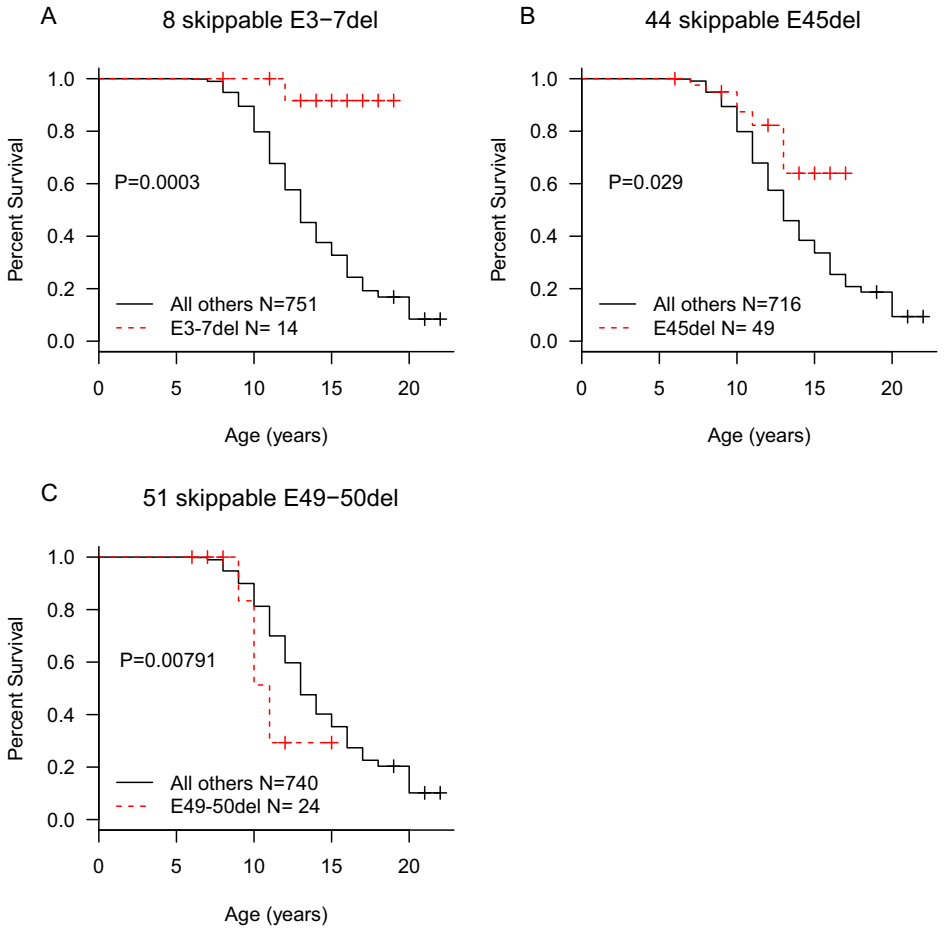

FIGURE 3 Kaplan–Meier plots for subgroups of exon 8, 44, and exon 51 skippable mutations. (A) Exon 8 skippable patients with exon 3–7 deletions ambulated substantially longer than any other group ($P = 0.0003$). (B) Individuals with single exon 45 deletions ambulate longer than other exon 44 skippable subgroups or other targeted exons ($P = 0.029$). (C) Among exon 51 skippable subgroups, only exon 49–50 deletions show significant change in age at LOA ($P = 0.008$)

(Table 2). Both variables had HR < 1, indicating an effect of delaying age at LOA. Moreover, exon 45 deletions were independently significant predictors of late age at LOA ($P = 0.015$, HR 0.49, CI 0.28–0.87) as well as exon 3–7 deletions ($P = 0.00125$, HR 0.18, CI 0.06–0.51) when included into the previous model. As expected, corticosteroid status was significant for both prednisone ($P = 0.0077$, HR 0.62, CI 0.44–0.88) and deflazacort ($P < 0.0001$, HR 0.30, CI 0.21–0.43), and of a similar effect size as exon 44 skippable mutation status. No other variables were significant.

Under the hypothesis that some DMD mutations may result in endogenous skipping to produce an in-frame mRNA, we quantitated exon skipping in myotube cultures derived from patient iDRM

TABLE 2 Cox regression results using age at LOA as outcome and corticosteroid status and mutation subgroup as covariates

	HR (CI low, high)	*P*
Current steroids (prednisone)	0.62 (0.44, 0.88)	**<0.01**
Current steroids (deflazacort)	0.31 (0.22, 0.43)	**<0.01**
Discontinued steroids	1.35 (0.94, 1.94)	0.10
Exon 8 skippable	0.21 (0.08, 0.53)	**<0.01**
Exon 44 skippable	0.54 (0.33, 0.87)	**0.01**
Exon 45 skippable	1 (0.65, 1.55)	0.99
Exon 50 skippable	0.8 (0.46, 1.36)	0.40
Exon 51 skippable	0.99 (0.67, 1.47)	0.96
Exon 52 skippable	1.02 (0.6, 1.72)	0.95
Exon 53 skippable	0.9 (0.59, 1.37)	0.62
Exon 55 skippable	0.92 (0.5, 1.68)	0.78
Duplication	0.99 (0.65, 1.49)	0.95

All variables were entered into a Cox regression model. Significant covariates with $P < 0.05$ are bolded. HR < 1 delays age at LOA. CI, confidence interval.

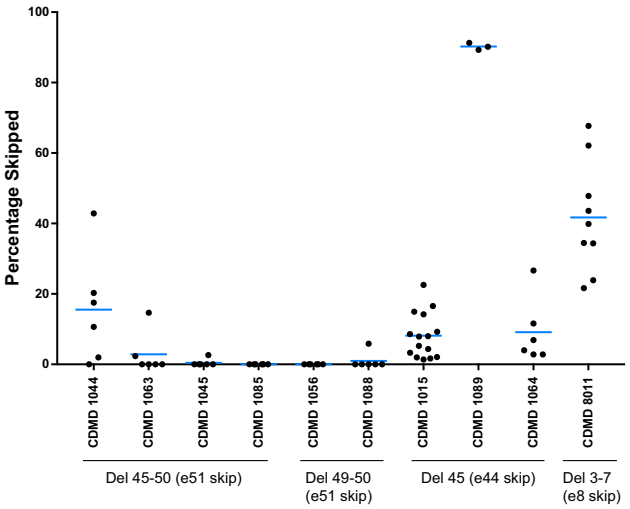

FIGURE 4 Basal levels of exon skipping are enriched in cultured myotubes derived from reprogrammed fibroblast (iDRM) from patients with del 45 mutations (exon 44 skippable) or myoblasts derived from del 3–7 (exon 8 skippable) relative to those derived from del 45–50 or del 49–50 iDRM (exon51 skippable). Experimental samples were run in triplicate and data shown reflect cumulative results of multiple experiments, with each point representing a singlet. *DMD* mRNA was reverse transcribed and PCR used to detect exon 44, 51, or 8 skipped and unskipped products. Products were quantitated using a Bioanalyzer. Percentage skipped is calculated as (skipped/unskipped + skipped) × 100

or myoblasts bearing mutations amenable to reframing by skipping exon 51, 45, 44, or 8 available through the CDMD tissue and cell repository. Of note, the representation of DMD mutations in the panel of myotubes examined are largely reflective of the frequencies found in the DMD population: patient mutations reframed by exon 51 skipping comprise the largest cohort, followed by those reframed by exon 45 skipping, exon 44 skipping, or exon 8 skipping, in order of decreasing frequency. We performed reverse transcription PCR on *DMD* RNA extracted from the myotubes and resulting amplicons were quantitated by capillary electrophoresis on an Agilent Bioanalyzer. Five of six iDRM cultures amenable to skipping of *DMD* exon 51 (Figure 4, e51 skip) showed less than 3% of exon 51 skipping, whereas one iDRM demonstrated a high frequency of skipping exon 51 (15%). iDRM lines harboring exon 45 deletions showed exon 44 skipping ranging from 8% to 90%. The myotube culture with an exon 8 skippable mutation also showed high endogenous exon skipping (43%).

In the one instance, where muscle biopsy was performed (CDMD8011), we assessed dystrophin protein levels in both frozen sections and expanded myoblasts fused to myotubes in culture. While deletion of exons 3–7 is predicted to result in a frameshift and loss of dystrophin protein, in keeping with high levels of endogenous skipping observed in the cultured patient myotubes (Figure 4), we found rescued dystrophin protein expression in both transverse sections of frozen muscle biopsy and cultured myotubes (Figure 5).

Our results are consistent with a model in which endogenous exon skipping in *DMD* transcripts results in a low level of in-frame mRNA and production of low levels of rescued dystrophin protein, which contributes toward reduction in disease severity as measured by delay in age of LOA in exon 44 or 8 skippable patients.

4 | DISCUSSION

We previously reported the utility of the Duchenne Registry patient registry to observe effects on age at LOA due to single and combination therapies for those affected by DMD (Wang et al., 2014). Furthermore, we demonstrated Duchenne Registry participants were typical of the general DMD population in terms of mutation status, mutation type, or age at diagnosis. Thus, this valuable dataset is highly applicable for observing differences in mutation type within *DMD* based on age at LOA and serving as a reference population demonstrating typical disease course.

The Duchenne Registry records current medication, mutation type, and age at LOA for a large number of participants, allowing us to observe differences in disease severity based on a limited group of mutations potentially amenable to exon skipping. As a web-based platform, the Duchenne Registry has a relatively low barrier of entry for participants, allowing for a large number of participants. In the available data from the Duchenne Registry, we were able to retain a substantial number ($N = 765$) of males with an exonic duplication, nonsense mutation, or deletion mutation correctable by skipping exon 8, 44, 45, 50, 51, 52, 53, or 55 alongside their steroid usage and age at LOA. The availability of these multiple data types and the ability of participants to update data make the Duchenne Registry a robust data set for exploratory studies and natural history comparison because it is larger than all reported studies investigating DMD age at LOA combined (Bello et al., 2016; Pane et al., 2014; Servais et al., 2015; van den Bergen, et al., 2014).

Our analysis of age at LOA supports the prior observations that not all mutations in the most commonly mutated region of *DMD* are equivalent, and that those amenable to exon 44 (Bello et al., 2016; Koeks et al., 2017; Pane et al., 2014; van den Bergen, et al., 2014) and exon 8 (Bello et al., 2016) skip therapy have a relatively mild disease

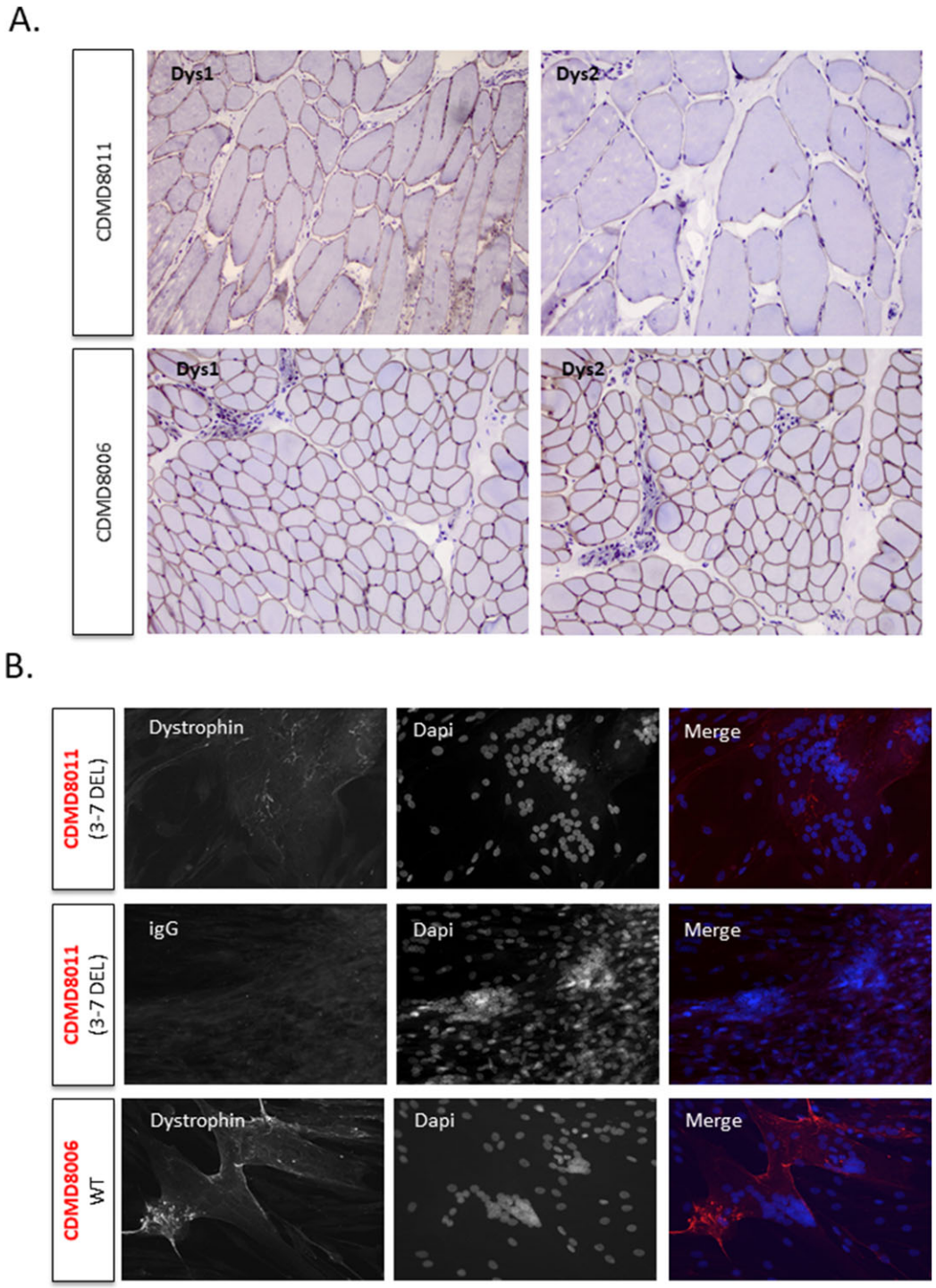

FIGURE 5 Patient CDMD8011 (del 3–7) expresses low levels of dystrophin protein in muscle biopsy and primary myoblasts expanded and fused to myotubes in culture. (A) Dystrophin is visible at the sarcolemma in transverse sections of muscle in CDMD8011 and CDMD8006 (wild-type) when stained with DysI (central rod domain) or Dys2 (C-terminal). Magnification, 10×, 20× for Dys2 CDMD8011. (B) Representative images of fused myotubes showed low amounts of dystrophin in CDMD8011 and higher levels in CDMD8006. Mandys8 stains the central rod domain of DMD. Nuclei are colored blue (Dapi). Scale bar 50 μm

phenotype, which impacts how clinical trials assessing therapeutic efficacy would need to be designed. In a study from multiple Italian centers, the subgroup eligible for exon 44 skipping had overall longer 6-minute walk distance (6MWD) than the other deletion mutations, but this result was not significant within a test of heterogeneity (Pane et al., 2014). The lack of statistical significance may be due to the relatively small number of individuals analyzed ($N = 18$ with exon 44 skippable mutations). Two studies from the United States and Netherlands studied age at LOA stratified by AON amenable treatment groups (Bello et al., 2016; van den Bergen et al., 2014). Consistent with the results presented here, both strongly support a delay in age at LOA among individuals amenable to targeted skipping of exon 44.

We estimated the effect of possessing a *DMD* mutation that is amenable to exon 44 skipping to have a hazard ratio (HR) of 0.54. This indicates that the impact of these mutations on the phenotype is roughly comparable with corticosteroid treatment. HRs for deflazacort and prednisone were 0.31 and 0.62, which is consistent with our previous finding that deflazacort tends to have a stronger effect at delaying age at LOA than prednisone (Wang et al., 2014). This is similar to Bello et al. (2016), who estimated HR for exon 44 skippable mutations to be 0.34. Their data also showed deflazacort to be more beneficial in delaying age at LOA compared to prednisone (0.34 vs. 0.22) and that the genetic effect was similar to corticosteroid treatment. These consistencies support the overall utility of the patient self-report registry model.

In the Duchenne Registry and other registries, exon 45 deletion mutations comprise 65% of DMD subjects who are potentially amenable to exon 44 skipping to restore *DMD* reading frame and represent 4% of all mutations that cause DMD (Aartsma-Rus et al., 2006; Tuffery-Giraud et al., 2009). We found exon 45 deletions were the primary driver of delayed age at LOA. When considering exon 45 deletions separately from other exon 44 skippable mutations, the HR in our Cox model decreased from 0.54 to 0.50 and only exon 45 deletions were significant ($P = 0.02$). Several studies have highlighted phenotypic heterogeneity of individuals with mutations around the exon 45–50 hot spot (Deburgrave et al., 2007; Kesari et al., 2008). One biological explanation is that more mildly affected patients may have a higher frequency of revertant fibers due to spontaneous skipping of an exon that generates in-frame transcripts and resulting functional dystrophin (Dwianingsih et al., 2014; Prior et al., 1997). Although an increased rate of revertant fibers has been demonstrated in some cases (Lourbakos et al., 2011), the true incidence is not known. We found evidence of endogenous exon skipping in patient-derived iDRMs and myotube cultures amenable to skipping of exons 8 and 44, and this provides a possible biological explanation for the effects observed in the Kaplan–Meier analysis. Baseline and repeat muscle biopsy studies in these patient subgroups with careful dystrophin quantification will shed more light on this as individuals are tested within the context of therapeutic trials intended to restore dystrophin to determine the phenotypic benefit of a low levels of dystrophin in humans. This could serve as a valuable model to determine the phenotypic benefit of low levels of dystrophin in humans. Additionally, iDRM-derived from DMD patients amenable to reframing by skipping exon 44, 51, or 8 provide culture models for elucidating the molecular basis of enhanced natural skipping and rescued dystrophin production. These would serve as useful adjuncts to ongoing concerns regarding dystrophin quantification.

Among the individuals potentially amenable to exon 8 skipping strategy, deletion of exons 3–7 was the most common mutation reported. HR was 0.21 in the larger group ($N = 18$) and 0.18 in those with exon 3–7 deletions ($N = 14$) and remained statistically significant ($P = 0.001$). Bello et al. estimated exon 3–7 deletions HR to be 0.24, demonstrating remarkable consistency in these sample sets. In both studies, the number of individuals with exon 8 skippable mutations is small, which may limit accurate measurement of the magnitude of effect. We have derived a cell line with deletion of exons 3–7 of *DMD* in which around 10% of *DMD* mRNA demonstrates endogenous skipping of exon 8, which is a potential molecular mechanism for why this group of patients is more mild.

Evidence of a more severe than typical disease course in the exon 51 amenable deletion group has not been reported elsewhere, and the molecular mechanism is not clear at this point. Kaplan–Meier analysis of individual subgroups within exon 51 amenable mutations revealed that exon 49–50 deletions appeared to be particularly severe with a median age at LOA of 11 years versus 13 years for the rest of the cohort ($P = 0.008$). However, this result was not significant in the Cox analysis.

Based on these findings, the exon 8, 44, and 51 skippable subgroups may serve as inappropriate natural history controls for most exon-skipping trials, but that other mutations amenable to exon 45, 50, 52, 53, 55, and some 51 or nonsense mutations are generally comparable and can reasonably serve as contemporary natural history controls. Even in instances where restoration of dystrophin is not the intended effect, the underlying genetic mutation may need to be considered to prevent the confounding of genetic effects within the potential therapeutic effect being sought. Thus, more natural history data may be necessary to improve the sensitivity of ongoing clinical trials for DMD in order to appropriately factor *DMD* mutation effects into clinical trial designs, and exact mutation type in the *DMD* gene should be considered in data interpretation. It is possible that restriction in clinical trial entry based on mutation type could reduce subject variability and enhance the ability to observe a study drug effect.

Because much of the Duchenne Registry data are based on patient and parent self-reports, there is sometimes concern regarding accuracy relative to natural history studies derived from academic medical centers where participants are recruited, phenotyped and followed longitudinally by expert clinicians. Although care has been taken to rule out inconsistencies and errors in responses (for instance, DMD genetic reports are reviewed and inputted by certified genetic counselors), misunderstandings and errors can occur in the survey questionnaire. We also note that the retrospective nature of this study and its internet-based recruitment methods may also influence the demographics of respondents, which may tend toward more technically literate families. However, the remarkable consistency of the findings reported here and the recently reported support for the observed superiority of deflazacort relative to prednisone (Griggs et al., 2016; Wang et al., 2014), serve to further validate and increase confidence in this important resource.

Using a large patient self-report registry, we have linked subgroups of genetic mutations to a critical and irreversible physiological milestone in DMD. These results indicate further study of exon 3–7 and exon 45 deletion subjects, which both show delayed age at LOA, will likely produce further understanding of the structure and function of aberrantly affected *DMD* transcripts and naturally occurring mitigating factors. Furthermore, the lack of equivalence in disease progression between different mutation subgroups that is apparent with the large sample set suggests caution when used for clinical trials or as natural history or external contemporary controls. However, the consistency of the age at LOA across multiple mutation types provides a powerful way to determine if long-term administration of new therapeutics is causing deviation from expected disease course. This will become increasingly important as drugs such as Exondys51 gain

approval through the accelerated approval pathway based on a reasonably likely to predict clinical benefit standard.

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CONFLICT OF INTEREST

The authors have no conflicts of interest to report.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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