Original Investigation

Biochemical Characterization of Patients With In-Frame or Out-of-Frame *DMD* Deletions Pertinent to Exon 44 or 45 Skipping

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IMPORTANCE In Duchenne muscular dystrophy (DMD), the reading frame of an out-of-frame *DMD* deletion can be repaired by antisense oligonucleotide (AO)–mediated exon skipping. This creates a shorter dystrophin protein, similar to those expressed in the milder Becker muscular dystrophy (BMD). The skipping of some exons may be more efficacious than others. Patients with exon 44 or 45 skippable deletions (AOs in clinical development) have a less predictable phenotype than those skippable for exon 51, a group in advanced clinical trials. A way to predict the potential of AOs is the study of patients with BMD who have deletions that naturally mimic those that would be achieved by exon skipping.

OBJECTIVE To quantify dystrophin messenger RNA (mRNA) and protein expression in patients with *DMD* deletions treatable by, or mimicking, exon 44 or 45 skipping.

DESIGN, SETTING, AND PARTICIPANTS Retrospective study of nondystrophic controls (n = 2), patients with DMD (n = 5), patients with intermediate muscular dystrophy (n = 3), and patients with BMD (n = 13) at 4 university-based academic centers and pediatric hospitals. Biochemical analysis of existing muscle biopsies was correlated with the severity of the skeletal muscle phenotype.

MAIN OUTCOMES AND MEASURES Dystrophin mRNA and protein expression.

RESULTS Patients with DMD who have out-of-frame deletions skippable for exon 44 or 45 had an elevated number of revertant and trace dystrophin expression (approximately 19% of control, using quantitative immunohistochemistry) with 4 of 9 patients presenting with an intermediate muscular dystrophy phenotype (3 patients) or a BMD-like phenotype (1 patient). Corresponding in-frame deletions presented with predominantly mild BMD phenotypes and lower dystrophin levels (approximately 42% of control) than patients with BMD modeling exon 51 skipping (approximately 80% of control). All 12 patients with in-frame deletions had a stable transcript compared with 2 of 9 patients with out-of-frame deletions (who had intermediate muscular dystrophy and BMD phenotypes).

CONCLUSIONS AND RELEVANCE Exon 44 or 45 skipping will likely yield lower levels of dystrophin than exon 51 skipping, although the resulting protein is functional enough to often maintain a mild BMD phenotype. Dystrophin transcript stability is an important indicator of dystrophin expression, and transcript instability in DMD compared with BMD should be explored as a potential biomarker of response to AOs. This study is beneficial for the planning, execution, and analysis of clinical trials for exon 44 and 45 skipping.

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Duchenne muscular dystrophy (DMD) is caused by frame-disrupting mutations in the *DMD* gene that prevent the full translation of its protein product, dystrophin.[1,2] Duchenne muscular dystrophy is characterized by progressive muscle weakness and degeneration resulting in loss of ambulation at approximately 13 years of age in the steroid-treated population[3] and death from respiratory and/or cardiac failure in the third or fourth decade of life when supported by noninvasive ventilation.[4-6] In-frame (IF) dystrophin mutations that do not disrupt the open reading frame typically cause a clinically milder disorder, Becker muscular dystrophy (BMD).[7,8] The IF mutations result in the translation of an internally deleted dystrophin protein; variations in the amount and functionality of these different dystrophin proteins contribute to the variable phenotypic spectrum of BMD.[9]

Dystrophin is a large sarcolemmal protein that forms part of the dystrophin-associated protein complex.[10,11] Dystrophin protects muscle fibers against the mechanical forces of contraction and plays a role in signaling; the loss of dystrophin leads to secondary sarcolemmal protein deficiencies.

Antisense oligonucleotide (AO)–mediated exon skipping aims to restore the *DMD* reading frame to allow the production of an internally deleted dystrophin protein and, hopefully, a functional benefit to patients with DMD who have out-of-frame (OOF) deletions.[12-14] The restoration of dystrophin and members of the dystrophin-associated protein complex has been achieved following local and systemic injections of AOs targeting exon 51.[15-19] Efforts are now focused on targeting other exons[12,20]; however, the skipping of some exons may be more efficacious than others. To predict the potential of various exon skipping strategies, we studied patients with BMD who have deletions that naturally mimic those achieved by exon skipping.

We previously quantified dystrophin in patients with BMD who have IF deletions that mimic skipping of exons 51, 45 through 55, and 53.[21] While these results proved encouraging for exon 51 and 53 skipping, several factors should be considered when developing and assessing the efficacy of strategies for skipping exons 44 and 45, which are among the most common *DMD* skippable exons. First, the percentage of revertant and trace dystrophin expression in patients with deletions flanking exon 44 is significantly higher than with deletions surrounding exon 51.[22] Second, OOF deletions around exons 44 and 45 result in a variety of clinical severities including intermediate muscular dystrophy (IMD) and BMD.[23-27]

Herein, we characterize patients with IF and OOF *DMD* deletions around exons 44 and 45 to enhance the planning, execution, and analysis of clinical trials for exon 44 or 45 skipping.

Methods

Twenty-one patients (**Table 1**) were retrospectively selected from 4 centers: (1) University College London, Institute of Child Health and Great Ormond Street Hospital, London, England; (2) Institute of Genetic Medicine, Newcastle University, Newcastle, England; (3) Institute of Neurology, Catholic University School of Medicine, Rome, Italy; and (4) University of Ferrara, Ferrara, Italy. Research ethics committee approval was obtained from the London–West London Gene Therapy Advisory Committee. Local research and development approval was also obtained from Great Ormond Street Hospital and the Institute of Child Health.

Selection criteria included an IF or OOF *DMD* deletion relevant to exon 44 or 45 skipping[28] (confirmed by multiplex ligation-dependent probe amplification) and the availability of a muscle biopsy. A standardized questionnaire was distributed to obtain information on genetic mutation, age at onset, age at biopsy, motor function abilities at the latest assessment, and other comorbidities.

Patients with BMD were classified as asymptomatic, having mild BMD, or having severe BMD according to the age at onset, relevant history, and overall motor function throughout the disease course. Asymptomatic individuals had no detectable muscle weakness and the only pathological feature was an elevated serum creatine kinase level. Mild BMD was defined as having mild proximal muscle weakness but retaining running ability beyond adolescence. Individuals who either lost running ability by the end of adolescence or never ran were classified as having severe BMD.

Skeletal muscle biopsies (14 quadriceps, 4 deltoid, and 3 unknown) were previously obtained with written informed consent. Nondystrophin control muscle biopsies (n = 2, paraspinal and intercostal muscles) were obtained from the MRC Centre for Neuromuscular Diseases Biobank, London (http://www.cnmd.ac.uk). Biochemical analysis of all samples was performed in London to minimize variability.

Quantitative immunohistochemistry was performed as previously described.[21] A 2-tailed unpaired *t* test was used for statistical analysis, and statistical significance was set at $P = .05$.

Western blotting was performed as previously described.[21] Dystrophin intensity was normalized to α-actinin using Image Laboratory software (BioRad) and expressed as a percentage of control.

Total RNA was extracted, DNase treated, reverse transcribed, and subjected to quantitative polymerase chain reaction as previously described.[29] Three dystrophin TaqMan assays (Applied Biosystems) were used with probes spanning the junctions of exons 19 and 20, 53 and 54, and 73 and 74 (**Table 2**). Starting concentrations were calculated using the LinRegPCR program.[30,31] Data were normalized to myotilin and presented relative to control. A 2-way analysis of variance and Bonferroni posttest were used to determine statistical significance.

Results

We studied a total of 21 patients (Table 1). Of these, 9 had OOF deletions skippable by exon 44 or 45 (models 44 and 45 OOF) and 12 had IF deletions mimicking the skipping of exon 45 (model 45 IF).

Clinical Characteristics

Four of 9 patients (44.4%) with OOF deletions had an IMD or BMD phenotype. Patients 1 and 2 (both with IMD) were am-

Table 1. Summary of Clinical Features

Patient No.	Exon Deletion	Exon Skipping Model	Frame	Age at Biopsy, y	Age at Onset	Phenotype	Symptoms	Motor Function
1	42-43	44	OOF	7	6 y	IMD	Unable to jump	Walking indoors at age 15 y
2	42-43	44	OOF	9	3 y	IMD	Motor delay	Walking indoors at age 14 y
3	45	44	OOF	8	Unknown	IMD	Toe walking	Ambulant at age 17 y, not running, difficulty climbing stairs
4	45	44	OOF	3	2 y	DMD	Frequent falls	LOA at age 11 y
5	45	44	OOF	1	17 mo	DMD	Delayed walking, diagnosed by incidental high serum CK level	LOA at age 12 y
6	44	45	OOF	17	Teens	BMD	...	Ambulant indoors at age 37 y
7	44	45	OOF	5	2 y	DMD	Difficulty walking	LOA at age 11 y
8	44	45	OOF	4	2 y	DMD	Delayed walking	LOA at age 11 y
9	46-47	45	OOF	5	4 y	DMD	Frequent falls, unable to jump	LOA at age 11 y
10	45-47	45	IF	8	5 y	BMD (mild)	Walking and learning difficulties	Ambulant at age 20 y, difficulty climbing stairs
11	45-47	45	IF	16	8 y	BMD (mild)	Difficulty walking	Ambulant at age 33 y, difficulty climbing stairs
12	45-47	45	IF	Unknown	Unknown	BMD (mild)	...	Ambulant at age 13 y
13	45-47	45	IF	7	6 y	BMD (mild)	Enlarged calves	Ambulant at age 9 y, swimming and sporty
14	45-47	45	IF	3	3 y	BMD (asymptomatic)	Incidental finding	Able to run at age 14 y, Gowers sign negative
15	45-48	45	IF	6	5 y	BMD (mild)	Difficulty walking	Able to run at age 19 y, plays football
16	45-48	45	IF	33	8 y	BMD (mild)	Difficulty climbing stairs	Ambulant at age 36 y
17	45-49	45	IF	7	5 y	BMD (severe)	Unable to jump, calf hypertrophy	Ambulant at age 17 y, wheelchair for long distances, struggles to rise from floor
18	45-49	45	IF	10	9 y	BMD (severe)	Calf hypertrophy, motor difficulties	Ambulant for maximum of 10 min at age 14 y, wheelchair for long distances
19	45-49	45	IF	3	3 y	BMD (severe)	Incidental finding, severely autistic	Ambulant at age 14 y, unable to run
20	45-49	45	IF	5	5 y	BMD (mild)	Motor and learning difficulties, behavioral problems	Ambulant at age 15 y, able to run, plays football
21	45-49	45	IF	6	1-2 y	BMD (mild)	Difficulty rising from floor	Ambulant at age 21 y

Abbreviations: BMD, Becker muscular dystrophy; CK, creatine kinase; DMD, Duchenne muscular dystrophy; IF, in-frame; IMD, intermediate muscular dystrophy; LOA, loss of ambulation; OOF, out-of-frame; ellipses, unreported.

bulant at ages 15 and 14 years, respectively, despite having discontinued steroids. Patients 3 (with IMD) and 6 (with BMD) are still ambulant at ages 17 and 37 years, respectively. All 12 patients with IF deletions had a BMD phenotype. Their average age at study was 9 years (range, 3-33 years) and the average age at onset was 5 years (range, 1-9 years). Of the 12 patients with IF deletions and BMD, 1 was asymptomatic, 8 had mild BMD, and 3 had severe BMD. Patient 14 with asymptomatic BMD was diagnosed incidentally at age 3 years and was able to run when last seen at the clinic at age 14 years. Patient 18 with severe BMD presented at age 9 years and by age 14 years he could not run and could only walk for a maximum of 10 minutes. Patients 17 and 19 with severe BMD had ages at onset of 5 and 3 years, respectively; while both retaining ambulation into their late teens, neither can run and patient 17 requires a wheelchair for long distances.

Table 2. TaqMan Assay Sequences

Assay	Primer		Probe	Product Length, bp	Mean PCR Efficiency[a]
	Forward	Reverse			
Dystrophin 19	(Exon 19) TCAGGCCCTGGTGGAACA	(Exon 20) CTGAGGCTTGTTTGATGCTATCTG	TGGTGAATGAGGGTGTTAA	65	1.81
Dystrophin 53	(Exon 53) GTCCCTATACAGTAGATGCAATCCAA	(Exon 54) GCCACTGGCGGAGGTCTT	CCAAGCAGTTGGCC	75	1.80
Dystrophin 73	(Exon 73) assay Hs01049401_m1[b]	(Exon 74) assay Hs01049401_m1[b]	Assay Hs01049401_m1[b]	105	1.90
Myotilin	(Exon 4) assay Hs00199016m1[b]	(Exon 5) assay Hs00199016m1[b]	Assay Hs00199016m1[b]	57	1.86

Abbreviations: bp, base pairs; PCR, polymerase chain reaction.

[a] Representative mean experimental efficiencies used to calculate starting concentration, NO. All assays had efficiencies greater than 90% when determined using the standard curve method on unlimited samples.

[b] Applied Biosystems catalog assay; exact sequences are unavailable.

Quantification of Dystrophin Protein and Transcript Expression

Dystrophin protein expression was quantified by immunohistochemistry using MANDYS106 (exon 43), MANEX50 (exons 49-50), and Dys2 (last 17 C-terminal amino acids) antibodies.

Dystrophin levels in the model 44 OOF group had a mean (SD) of approximately 20% (18.3%) of control for MANDYS106, approximately 25% (14.4%) for MANEX50, and approximately 16% (6.4%) for Dys2 (**Figure 1**A and eFigure 1 in Supplement). Patients in the model 45 OOF group had comparable mean (SD) levels of approximately 18% (20.2%) of control for MANDYS106, approximately 21% (24.3%) for MANEX50, and approximately 22% (26.0%) for Dys2. Higher levels of dystrophin expression were observed in the model 45 IF group, which had a mean (SD) of approximately 44% (6.8%) of control for MANDYS106, approximately 41% (8.8%) for MANEX50, and approximately 43% (9.2%) for Dys2 (Figure 1A). Patients 1 and 2 lack the MANDYS106 epitope and patients 17 through 21 lack the MANEX50 epitope; these data points were excluded as they do not represent dystrophin content.

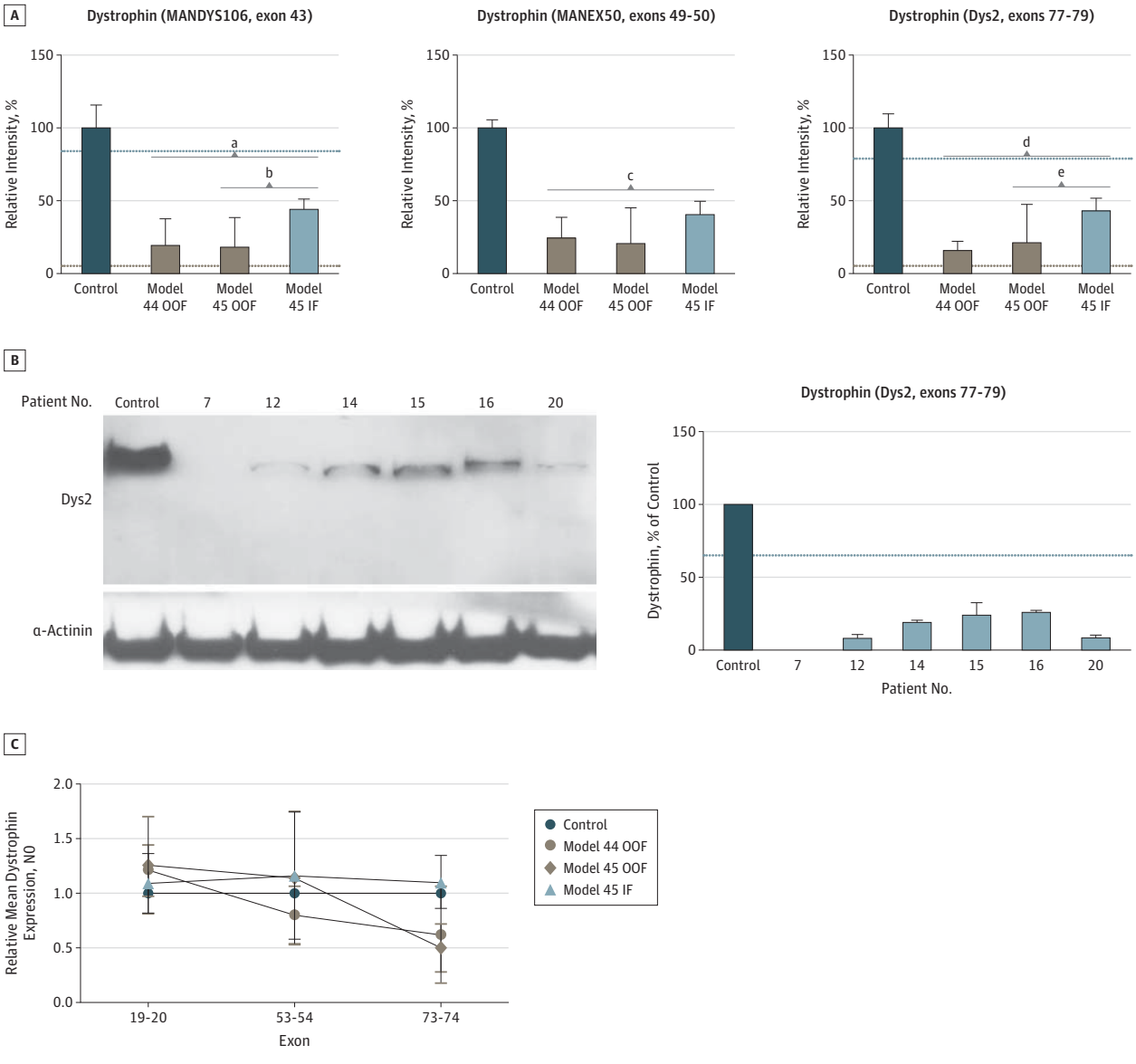
Notable variability was observed between patients with OOF and IF deletions (Figure 1A and eFigure 1 in Supplement). Patients 3 (with IMD) and 6 (with BMD) had relatively high dystrophin levels (approximately 41.0% and 48.4%, respectively, for MANDYS106), while patients 4 and 5 with DMD had low levels of dystrophin expression (approximately 9.1% and 8.8%, respectively, for MANDYS106). Mean dystrophin levels for the model 45 IF group were significantly higher than at least 1 of the OOF groups with all 3 antibodies (Figure 1A).

We compared the mean level of dystrophin protein expression in the IF and OOF groups vs patients with IF and OOF deletions from our previous study on patients with deletions relevant to exon 51 skipping (Figure 1A).[21] The level of dystrophin in OOF models 44 and 45 was higher than with OOF deletions skippable for exon 51. Conversely, the model 45 IF group had a lower dystrophin level (mean [SD], 44% [6.8%] for MANDYS106 and 43% [9.2%] for Dys2) than that previously reported for the IF model 51 group (mean [SD], 84% [15.8%] for MANDYS106 and 79% [17.1%] for Dys2).

We next quantified dystrophin protein expression in 6 patients (patient 7 with DMD and patients 12, 14, 15, 16, and 20 with BMD) by Western blotting (Figure 1B). No dystrophin was detected for patient 7 with DMD, while the patients with BMD had a mean (SD) dystrophin protein expression of 17% (7.5%) of control. In our previous study on patients with BMD mimicking exon 51 skipping, Western blotting revealed a dystrophin level of approximately 65% of control[21] (Figure 1B), confirming that dystrophin protein expression in this current BMD cohort is relatively low.

It has been suggested that it is the stability rather than the amount of the *DMD* transcript that determines the level of dystrophin protein and that there is a 5′ to 3′ imbalance in the stability of the dystrophin transcript.[32-35] We quantified dystrophin messenger RNA (mRNA) and investigated, indirectly, the stability of the dystrophin transcript using TaqMan assays spanning exon junctions 19 and 20, 53 and 54, and 73 and 74 (Figure 1C and eFigure 2 in Supplement). The relative expression of dystrophin mRNA using a TaqMan assay for the 5′ end of the transcript was similar to control in all 21 patients and the level of dystrophin mRNA does not correlate to the level of dystrophin protein (Figure 1, and eFigure 1 and eFigure 2 in Supplement). We observed a decrease in transcript levels in some patients with the exon junction 53 and 54 assay (ie, 3′ to the deletion breakpoint) that was more pronounced in patients with OOF deletions. Model 45 OOF and model 45 IF group means were statistically different with the exon junction 73 and 74 probe (P = .03) (Figure 1C), with 7 of 9 patients in the OOF group (patients 1, 2, 4, 5, 7, 8, and 9) showing a clear *DMD* transcript instability (eFigure 2 in Supplement). Interestingly, all these patients have been diagnosed as having DMD or IMD and low dystrophin levels compared with patients with a more stable dystrophin transcript. Patients 3 and 6 with IMD were the only 2 patients with OOF deletions that retained a high level of *DMD* mRNA expression with the exon junction 73 and 74 assay (mean [SD], approximately 1.35 [0.30] and 0.83 [0.08], respectively); these patients also had the highest dystrophin protein levels among the OOF cohort (mean [SD], approximately 40.6% [10.66%] and 48.4% [20.07%], respectively, with MANDYS106). Similarly, all patients with BMD who have IF deletions retained a high level of *DMD* mRNA with all 3 assays (mean [SD], approximately 1.09 [0.27] for the exon junction 19 and 20 assay, approximately 1.16 [0.58] for the exon junction 53 and 54 assay, and approximately 1.10 [0.24] for the exon junction 73 and 74 assay) (Figure 1C).

Figure 1. Comparative Analysis of Dystrophin Protein and Transcript Expression in Patients With In-Frame or Out-of-Frame *DMD* Deletions Around Exons 44 and 45



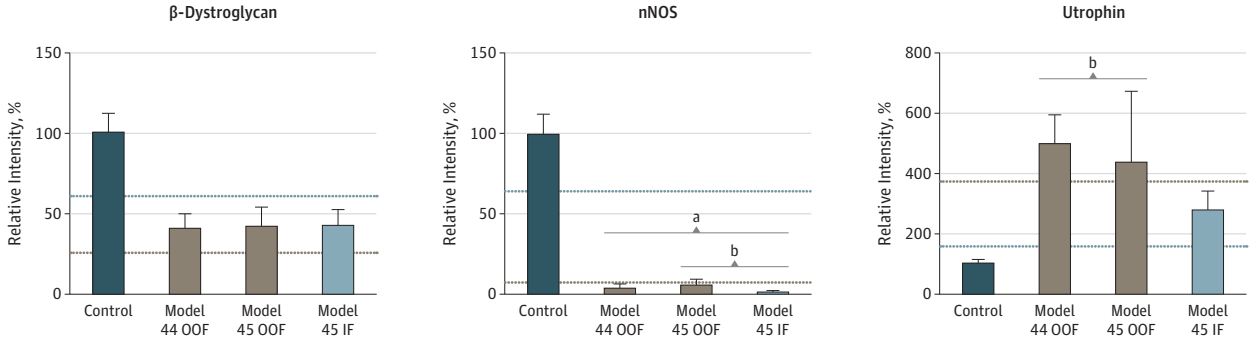
A, Transverse muscle sections were immunolabeled for β-spectrin, MANDYS106, MANEX50, and Dys2. Protein expression was quantified relative to control muscle in 40 muscle fibers and normalized to β-spectrin expression. Patients were grouped according to corresponding exon skipping models for Duchenne muscular dystrophy: control, exon 44 or 45 skippable out-of-frame (OOF) deletions (model 44 OOF and model 45 OOF), and in-frame (IF) deletions mimicking exon 45 skipping (model 45 IF). Data are presented as mean (SD) of the difference between sample means. Dotted lines indicate the mean dystrophin protein expression level from patients with Becker muscular dystrophy who have IF (blue lines) and OOF (gray lines) deletions modeling exon 51 skipping quantified in our previous study.[21] B, Western blotting analysis of patients 7, 12, 14, 15, 16, and 20. Data are normalized to α-actinin and presented as the mean (SD) percentage of control. Dotted line indicates the mean dystrophin protein expression level from patients with Becker muscular dystrophy who have IF deletions modelling exon 51 skipping quantified in our previous study.[21] C, The *DMD* messenger RNA transcript levels were quantified using 3 separate TaqMan assays (Applied Biosystems) targeting exon boundaries 19 and 20, 53 and 54, and 73 and 74. The mean polymerase chain reaction efficiency per amplicon (Table 2) and the mean threshold cycle (Ct) value per sample were used to calculate the starting concentration (N0) using the equation $N0 = N_t/E^{Ct}$ where N_t is the fluorescence threshold and E is the efficiency. Data were normalized to myotilin, and dystrophin transcript expression is presented as mean (SD) relative to control.

[a] $P = .002$.
[b] $P = .001$.
[c] $P = .03$.
[d] $P < .001$.
[e] $P = .02$.

Quantification of Dystrophin-Associated Protein Expression

To assess the functional properties of the different dystrophin proteins expressed in our cohort, we quantified the expression of β-dystroglycan, neuronal nitric oxide synthase (nNOS), and utrophin by quantitative immunohistochemistry (**Figure 2** and eFigure 3 in Supplement).

Figure 2. Comparative Immunohistochemical Analysis of Dystrophin-Associated Protein Expression in Patients With In-Frame or Out-of-Frame *DMD* Deletions Around Exons 44 and 45



Transverse muscle sections were immunolabeled for β-spectrin, β-dystroglycan, neuronal nitric oxide synthase (nNOS), and utrophin. Expression was quantified relative to control muscle in 40 muscle fibers and normalized to β-spectrin expression. Patients were grouped according to corresponding exon skipping models for Duchenne muscular dystrophy: control, exon 44 or 45 skippable out-of-frame (OOF) deletions (model 44 OOF and model 45 OOF), and in-frame (IF) deletions mimicking exon 45 skipping (model 45 IF). Data are presented as mean (SD) of the difference between sample means. Dotted lines indicate the mean dystrophin-associated protein expression level from patients with Becker muscular dystrophy who have IF (blue lines) and OOF (gray lines) deletions modeling exon 51 skipping.[21]

[a] $P = .01$.

[b] $P < .001$.

The mean (SD) β-dystroglycan levels in the OOF and IF groups were lower than control and highly comparable (model 44 OOF: approximately 41% [8.9%] of control; model 45 OOF: approximately 42% [12.0%] of control; and model 45 IF: approximately 43% [9.9%] of control). These levels appear unrelated to the level of dystrophin protein expression (Figure 1 and eFigure 1 in Supplement). β-Dystroglycan levels in patients with IF deletions were lower than those with IF deletions mimicking exon 51 skipping (Figure 2), reflecting the lower level of dystrophin observed in this study; conversely, the β-dystroglycan levels in the OOF groups were higher than in patients with OOF deletions mimicking exon 51 skipping (Figure 2).[21]

All but 1 (patient 9) of the 21 patients have incomplete nNOS-binding domains (encoded by exons 42-45); consequently, these patients have virtually absent levels of nNOS expression, with patient 9 having the highest level of 8.8%. Sarcolemmal nNOS protein expression was significantly lower with the model 45 IF group compared with both OOF groups (Figure 2).

There is limited information on the levels of the dystrophin homolog utrophin in BMD or to what extent utrophin and dystrophin are present together at the sarcolemma.[36-38] The 2 OOF models had high mean (SD) levels of sarcolemmal utrophin expression (model 44 OOF: approximately 501% [94.7%] of control; and model 45 OOF: approximately 438% [235.1%] of control). The model 45 IF group had a significantly lower and less variable mean (SD) level of approximately 280% (63.5%) of control, which was higher than in patients with BMD modeling exon 51 skipping (mean [SD], approximately 159% [46.3%] of control) ($P < .001$) (Figure 2). We did not identify a relationship between the levels of dystrophin and utrophin. Patient 3 with IMD had among the highest sarcolemmal dystrophin and utrophin levels (mean [SD], approximately 41% [10.7%] with MANDYS106 and 540% [202.3%] of control, respectively); however, a similar level of dystrophin expression in patient 6 with IMD (mean [SD], approximately 48% [20.1%] of control) was accompanied by a lower level of sarcolemmal utrophin (mean [SD], approximately 182% [100.5%] of control) (Figure 1 and eFigure 3 in Supplement).

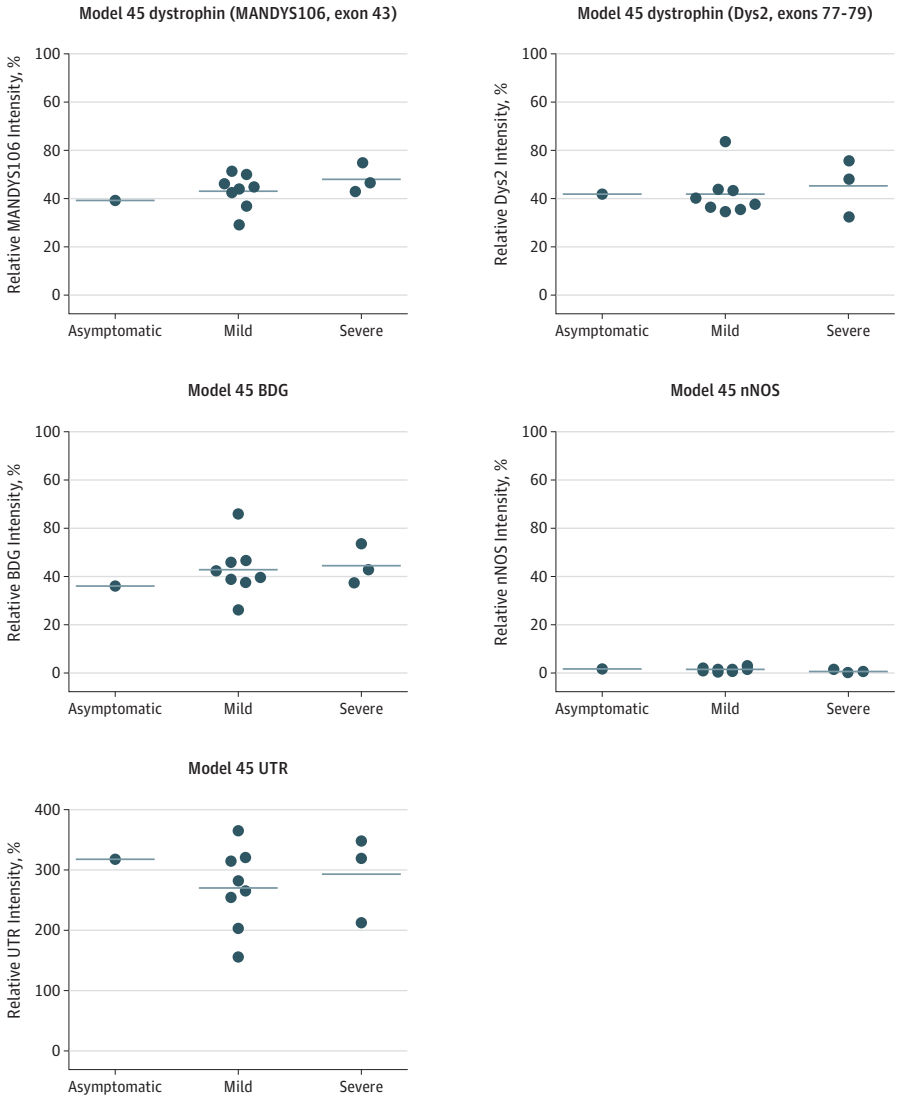
We found no obvious difference in the mean or spread of dystrophin or dystrophin-associated protein expression between patients with BMD of different severities (eFigure 1 and eFigure 3 in Supplement). We previously demonstrated that the levels of dystrophin and some dystrophin-associated proteins are correlated with clinical severity in a BMD cohort mimicking exon 51 skipping.[21] Herein, we found no clear correlation between dystrophin, β-dystroglycan, nNOS, or utrophin protein expression and clinical severity for the model 45 group. Asymptomatic patients, those with mild BMD, and those with severe BMD have comparable dystrophin and dystrophin-associated protein levels (**Figure 3**).

Discussion

We have quantitatively assessed the levels of dystrophin transcript and protein in a cohort of 21 patients with IF and OOF deletions around exons 44 and 45. We compared these data with our previous study[21] to provide the most comprehensive characterization of patients with BMD mimicking exon skipping to date. Our data provide a robust baseline for the assessment of dystrophin transcript and protein levels in boys with DMD recruited into future clinical trials.

Patients in the model 44/45 OOF group had higher levels of dystrophin protein than those modeling exon 51 or 53 skipping. We attribute this to a higher level of revertant and trace dystrophin expression in patients in the model 44/45 OOF group.[22,39] Exon 44 skips spontaneously when surrounding exons are deleted[40]; patients with these "leaky" mutations pre-

Figure 3. Correlation of Dystrophin and Dystrophin-Associated Protein Expression With Clinical Severity in Patients With In-Frame *DMD* Deletions Around Exons 44 and 45



Patients with Becker muscular dystrophy were grossly grouped as having asymptomatic, mild, or severe Becker muscular dystrophy according to the severity of their skeletal muscle phenotype. Lines represent the mean expression in each group. BDG indicates β-dystroglycan; nNOS, neuronal nitric oxide synthase; and UTR, utrophin.

sent with unpredictable phenotypes, which highlights the limitation of an exclusively genetic diagnosis and the value of accurate muscle pathology. Indeed, 4 of 9 patients in our OOF cohort had IMD or BMD phenotypes instead of the expected DMD phenotype. This is in stark contrast to OOF deletions further 3′ around exon 51, which predictably present as DMD.[21,41] Although in vitro data suggest that patients with leaky mutations might be ideal candidates for exon skipping,[39] the consequences of the higher background levels of dystrophin and the lower level of dystrophin protein restoration that could be achieved by exon skipping in these patients are unknown. The efficacy and efficiency of exon 44 and 45 AOs will thus be difficult to measure without pretreatment biopsies and sensitive quantitative methods. Phase I and II clinical trials of exon 44 and 45 AOs are under way (ClinicalTrials.gov identifiers NCT01037309 and NCT01826474, respectively); in light of the discussed data, patients with different treatment responses in these trials are likely. The higher level of dystrophin expression in patients eligible for exon 44 or 45 trials may influence their immunity to dystrophin.[42] Immunity to self- and nonself-dystrophin epitopes requires further investigation and should be assessed in future DMD clinical trials.

In our study, recruitment may be biased toward patients with severe BMD as many patients with asymptomatic BMD pass undiagnosed. On this note, we could not recruit any patients with BMD who have deletions mimicking exon 44 skipping; thus, our cohort cannot represent the full spectrum of deletions in this area, some of which have never been reported. Nevertheless, all patients with IF deletions in this study had dystrophin protein levels approximately 40% of control and 9 of 12 patients had mild BMD or were asymptomatic. Although our sample size is too small for meaningful analysis, we considered our previous cohort of IF deletions around exon 51 less severe (and the dystrophin protein more stable and/or

functional) as only 1 of 17 patients had severe BMD, while 4 of 17 were asymptomatic.[21]

While a number of disease modifiers have been identified for DMD,[43,44] no information is available on whether they contribute to BMD variability. Variability in BMD is likely affected by the structure and stability of dystrophin. Our 12 patients with BMD maintain an intact hinge 3, which results in more variable BMD phenotypes.[45] The phasing of helical repeats in patients with deletions in exons 45 to 47 and 45 to 49 is disrupted[46]; in our study, there are patients with asymptomatic, mild, and severe BMD with out-of-phase repeats.

We observed no clear correlation between the level of dystrophin transcript or protein expression with clinical severity in patients with IF deletions modeling exon 45 skipping. This is in concordance with recently presented protein data on 13 patients with BMD who have a deletion of exons 45 to 47.[47]

Antisense oligonucleotide–mediated exon skipping relies on the availability of the dystrophin transcript. Our finding that patients with IF and OOF deletions have 5′ *DMD* transcript expression levels comparable to control is supported by studies suggesting that transcript stability rather than amount is important for high levels of dystrophin protein expression.[35,48] We show that all 12 patients with IF deletions had a stable transcript, while all patients with an OOF deletion (accompanied by a low level of dystrophin expression) had an unstable dystrophin transcript. This is supported by another study that showed a 5′ to 3′ increase in threshold cycle values in patients with DMD who have OOF deletions and point mutations.[49] Taken together, these data highlight the need for careful consideration of the location of primers and probes when quantifying dystrophin mRNA and suggest that transcript stability correlates with protein production better than overall transcript levels. Whether exon skipping restores the stability of the dystrophin transcript in vivo and how existing patient transcript instabilities (which may vary according to the OOF mutation) affect the outcome of exon skipping remain to be determined. The assessment of *DMD* transcript stability could be explored as a marker of response to therapy. Recent data show that the transcript imbalance is more pronounced in mdx mice harboring a nonsense mutation vs wild type.[35] Thus, a strategy to restore and maintain *DMD* transcript stability may improve the efficiency of AO-mediated exon skipping.

In summary, our data suggest that, as with exon 51, AO-mediated exon skipping of exon 44 or 45 may prove beneficial to patients with DMD, as patients with IF mutations in this region had a predominantly mild BMD phenotype. However, we highlight that the skipping of different exons will likely yield different levels of dystrophin protein restoration as well as dystrophin proteins that differ in their stability and functionality.

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