**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.
Duchenne muscular dystrophy (DMD) is caused by frame-disrupting mutations in the DMD gene that prevent the full translation of its protein product, dystrophin. 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 and death from respiratory and/or cardiac failure in the third or fourth decade of life when supported by noninvasive ventilation. In-frame (IF) dystrophin mutations that do not disrupt the open reading frame typically cause a clinically milder disorder, Becker muscular dystrophy (BMD). 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.

Dystrophin is a large sarcolemmal protein that forms part of the dystrophin-associated protein complex. Dystrophin protects muscle fibers against the mechanical forces of contraction and plays a role in signaling; the loss of dystrophin protects muscle fibers against the mechanical forces of contraction. 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. 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. Efforts are now focused on targeting other exons; 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. While these results proved encouraging for exon 51 and 52 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. Second, OOF deletions around exons 44 and 45 result in a variety of clinical severities including intermediate muscular dystrophy (IMD) and BMD.

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 (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.cmnd.ac.uk). Biochemical analysis of all samples was performed in London to minimize variability.

Quantitative immunohistochemistry was performed as previously described. 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. 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. 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. 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-
bulant at ages 15 and 14 years, respectively, despite having dis-
continued steroids. Patients 3 (with IMD) and 6 (with BMD) are
still ambulant at ages 17 and 37 years, respectively. All 12 pa-
tients 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>
<thead>
<tr>
<th>Patient No.</th>
<th>Exon Deletion</th>
<th>Exon Skipping Model</th>
<th>Frame</th>
<th>Age at Biopsy, y</th>
<th>Age at Onset, y</th>
<th>Phenotype</th>
<th>Symptoms</th>
<th>Motor Function</th>
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<tr>
<td>1</td>
<td>42-43</td>
<td>44</td>
<td>OOF</td>
<td>7</td>
<td>6</td>
<td>IMD</td>
<td>Unable to jump</td>
<td>Walking indoors at age 15 y</td>
</tr>
<tr>
<td>2</td>
<td>42-43</td>
<td>44</td>
<td>OOF</td>
<td>9</td>
<td>3</td>
<td>IMD</td>
<td>Motor delay</td>
<td>Walking indoors at age 14 y</td>
</tr>
<tr>
<td>3</td>
<td>45</td>
<td>44</td>
<td>OOF</td>
<td>8</td>
<td>Unknown</td>
<td>IMD</td>
<td>Toe walking</td>
<td>Ambulant at age 17 y, not running, difficulty climbing stairs</td>
</tr>
<tr>
<td>4</td>
<td>45</td>
<td>44</td>
<td>OOF</td>
<td>3</td>
<td>2</td>
<td>DMD</td>
<td>Frequent falls</td>
<td>LOA at age 11 y</td>
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<tr>
<td>5</td>
<td>45</td>
<td>44</td>
<td>OOF</td>
<td>1</td>
<td>17 mo</td>
<td>DMD</td>
<td>Delayed walking, diagnosed by incidental high serum CK level</td>
<td>LOA at age 12 y</td>
</tr>
<tr>
<td>6</td>
<td>44</td>
<td>45</td>
<td>OOF</td>
<td>17</td>
<td>Teens</td>
<td>BMD</td>
<td>...</td>
<td>Ambulant indoors at age 37 y</td>
</tr>
<tr>
<td>7</td>
<td>44</td>
<td>45</td>
<td>OOF</td>
<td>5</td>
<td>2</td>
<td>DMD</td>
<td>Difficulty walking</td>
<td>LOA at age 11 y</td>
</tr>
<tr>
<td>8</td>
<td>44</td>
<td>45</td>
<td>OOF</td>
<td>4</td>
<td>2</td>
<td>DMD</td>
<td>Delayed walking</td>
<td>LOA at age 11 y</td>
</tr>
<tr>
<td>9</td>
<td>46-47</td>
<td>45</td>
<td>OOF</td>
<td>5</td>
<td>4</td>
<td>DMD</td>
<td>Frequent falls, unable to jump</td>
<td>LOA at age 11 y</td>
</tr>
<tr>
<td>10</td>
<td>45-47</td>
<td>45</td>
<td>IF</td>
<td>8</td>
<td>5</td>
<td>BMD (mild)</td>
<td>Walking and learning difficulties</td>
<td>Ambulant at age 20 y, difficulty climbing stairs</td>
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<tr>
<td>11</td>
<td>45-47</td>
<td>45</td>
<td>IF</td>
<td>16</td>
<td>8</td>
<td>BMD (mild)</td>
<td>Difficulty walking</td>
<td>Ambulant at age 33 y, difficulty climbing stairs</td>
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<td>12</td>
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<td>45</td>
<td>IF</td>
<td>Unknown</td>
<td>Unknown</td>
<td>BMD (mild)</td>
<td>...</td>
<td>Ambulant at age 13 y</td>
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<tr>
<td>13</td>
<td>45-47</td>
<td>45</td>
<td>IF</td>
<td>7</td>
<td>6</td>
<td>BMD (mild)</td>
<td>Enlarged calves</td>
<td>Ambulant at age 9 y, swimming and sporting</td>
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<td>IF</td>
<td>3</td>
<td>3</td>
<td>BMD (asymptomatic)</td>
<td>Incidental finding</td>
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<td>45-48</td>
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<td>IF</td>
<td>6</td>
<td>5</td>
<td>BMD (mild)</td>
<td>Difficulty walking</td>
<td>Able to run at age 19 y, plays football</td>
</tr>
<tr>
<td>16</td>
<td>45-48</td>
<td>45</td>
<td>IF</td>
<td>33</td>
<td>8</td>
<td>BMD (mild)</td>
<td>Difficulty climbing stairs</td>
<td>Ambulant at age 36 y</td>
</tr>
<tr>
<td>17</td>
<td>45-49</td>
<td>45</td>
<td>IF</td>
<td>7</td>
<td>5</td>
<td>BMD (severe)</td>
<td>Unable to jump, calf hypertrophy</td>
<td>Ambulant at age 17 y, wheelchair for long distances, struggles to rise from floor</td>
</tr>
<tr>
<td>18</td>
<td>45-49</td>
<td>45</td>
<td>IF</td>
<td>10</td>
<td>9</td>
<td>BMD (severe)</td>
<td>Calf hypertrophy, motor difficulties</td>
<td>Ambulant for maximum of 10 min at age 14 y, wheelchair for long distances</td>
</tr>
<tr>
<td>19</td>
<td>45-49</td>
<td>45</td>
<td>IF</td>
<td>3</td>
<td>3</td>
<td>BMD (severe)</td>
<td>Incidental finding, severely autistic</td>
<td>Ambulant at age 14 y, unable to run</td>
</tr>
<tr>
<td>20</td>
<td>45-49</td>
<td>45</td>
<td>IF</td>
<td>5</td>
<td>5</td>
<td>BMD (mild)</td>
<td>Motor and learning difficulties, behavioral problems</td>
<td>Ambulant at age 15 y, able to run, plays football</td>
</tr>
<tr>
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<td>45-49</td>
<td>45</td>
<td>IF</td>
<td>6</td>
<td>1-2</td>
<td>BMD (mild)</td>
<td>Difficulty rising from floor</td>
<td>Ambulant at age 21 y</td>
</tr>
</tbody>
</table>

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.
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 1A 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, with MANDYS106). Similarly, 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.

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).23 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 control23 (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).

### Table 2. TaqMan Assay Sequences

<table>
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<tr>
<th>Assay</th>
<th>Primer</th>
<th>Probe</th>
<th>Product Length, bp</th>
<th>Mean PCR Efficiency</th>
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<tr>
<td>Dystrophin 19</td>
<td>(Exon 19) TACGCGCGCCGGGAAACA</td>
<td>TGGTGAATGAGGGGTATTTAA</td>
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<td>1.81</td>
</tr>
<tr>
<td>Dystrophin 53</td>
<td>(Exon 53) GTCCATATACGATGCAATCCA</td>
<td>(Exon 54) GCTGGCCGGAGCTTTT</td>
<td>75</td>
<td>1.80</td>
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<tr>
<td>Dystrophin 73</td>
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<td>Assay Hs01049401_m1a</td>
<td>105</td>
<td>1.90</td>
</tr>
<tr>
<td>Myotilin</td>
<td>(Exon 4) assay Hs00199016m1b</td>
<td>Assay Hs00199016m1b</td>
<td>57</td>
<td>1.86</td>
</tr>
</tbody>
</table>

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

* Represents mean experimental efficiencies used to calculate starting concentration, N0. All assays had efficiencies greater than 90% when determined using the standard curve method on unlimited samples.

* Applied Biosystems catalog assay; exact sequences are unavailable.
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 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 deletions modelling exon 51 skipping quantified in our previous study.27 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 (NO) using the equation \( N_0 = N_f/E^{C_t} \) where \( N_f \) 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.

\[ \text{p} = .002. \]
\[ \text{p} = .001. \]
\[ \text{p} = .03. \]
\[ \text{p} = .001. \]
\[ \text{p} = .02. \]

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).
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

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 study21 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 deleted40; patients with these “leaky” mutations pre-
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
patients with BMD who have a deletion of exons 45 to 47. This is in concordance with recently presented protein data on 13 trophin transcriptor protein expression with clinical severity.

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 47.46,47 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

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.

ARTICLE INFORMATION
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Author Contributions: Dr Anthony had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Anthony, Arechavala-Gomeza, Ricotti, Torelli, Feng, Bushby, Morgan, Muntoni. Acquisition of data: Anthony, Arechavala-Gomeza, Ricotti, Torelli, Feng, Janghra, Tasca, Guglieri, Barresi, Armaroli, Bushby, Straub, Ricci, Muntoni. Analysis and interpretation of data: Anthony, Arechavala-Gomeza, Ricotti, Torelli, Janghra, Tasca, Ferlini, Sewry, Morgan, Muntoni. Drafting of the manuscript: Anthony, Arechavala-Gomeza, Ricotti, Feng, Armaroli, Morgan, Muntoni. Critical revision of the manuscript for important intellectual content: Anthony, Arechavala-Gomeza, Ricotti, Torelli, Janghra, Tasca, Guglieri, Barresi, Ferlini, Bushby, Straub, Ricci, Sewry, Morgan, Muntoni. Statistical analysis: Anthony. Obtained funding: Ferlini, Straub, Morgan, Muntoni. Administrative, technical, or material support: Arechavala-Gomeza, Feng, Janghra, Barresi, Armaroli, Sewry. Study supervision: Anthony, Ricotti, Ferlini, Bushby, Straub, Morgan, Muntoni.

Conflict of Interest Disclosures: Dr Ferlini is principal investigator of 2 ongoing MD AO clinical trials (GlaxoSmithKline DMD114349 and Prosensa PRO044). Dr Bushby has served on scientific advisory boards for Acceleron, Amsterdam Molecular Therapeutics, Avi BioPharma, Debiopharm Group, Genzyme, GlaxoSmithKline, Prosensa, PTC Therapeutics, and Santhera Pharmaceuticals and has received funding for trials from Avi BioPharma and PTC Therapeutics. Dr Straub is a member of the scientific advisory boards of Genzyme, Acceleron, and Prosensa; has delivered lectures for Genzyme for which Newcastle University receives remuneration; and has received funding for a trial from GlaxoSmithKline. Dr Muntoni has served on scientific advisory boards for Acceleron Pharma, Genzyme, Avi BioPharma, Debiopharma Group, GlaxoSmithKline, Prosensa, Servier, and Santhera Pharmaceutical; serves on the editorial boards of Neuromuscular Disorders and Neuropediatrics; receives research support from the European Union, the Medical Research Council, the Wellcome Trust, the Association Française contre les Myopathies, the Muscular Dystrophy Campaign, the Great Ormond Street Hospital Biomedical Research Centre, and the Muscular Dystrophy Association; is receiving funding for trials from GlaxoSmithKline, Trophos, and the British Heart Foundation; and has received funding for trials from Avi BioPharma and PTC Therapeutics. No other disclosures were reported.

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