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Dystrophin levels and clinical severity in Becker muscular dystrophy patients

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Abstract

Objective

Becker muscular dystrophy (BMD) is characterized by broad clinical variability. Ongoing studies exploring dystrophin restoration in Duchenne muscular dystrophy ask for better understanding of the relationship between dystrophin levels and disease severity. We studied this relationship in BMD patients with varying mutations, including a large subset with an exon 45-47 deletion.

Methods

Dystrophin was quantified by Western blot analyses in a fresh muscle biopsy of the anterior tibial muscle. Disease severity was assessed using quantitative muscle strength measurements and functional disability scoring. MRI of the leg was performed in a subgroup to detect fatty infiltration.

Results

Thirty-three BMD patients participated. No linear relationship was found between dystrophin levels (range 3-78%) and muscle strength or age at different disease milestones, in both the whole group and the subgroup of exon 45-47 deleted patients. However, patients with less than 10% dystrophin all showed a severe disease course. No relationship was found between disease severity and age when analysing the whole group. By contrast, in the exon 45-47 deleted subgroup muscle strength and levels of fatty infiltration were significantly correlated with patients' age.

Conclusions

Our study shows that dystrophin levels appear not to be a major determinant of disease severity in BMD, as long as it is above approximately 10%. A significant relationship between age and disease course was only found in the exon 45-47 deletion subgroup. This suggests that at higher dystrophin levels the disease course depends more on the mutation site, than on the amount of the dystrophin protein produced.

Introduction

Becker and Duchenne muscular dystrophies (B/DMD) are caused by mutations in the *DMD* gene encoding the dystrophin protein. Absence of dystrophin, as in DMD patients, or reduced levels of a semi-functioning dystrophin, as in BMD patients, leads to progressive muscle weakness due to continuous muscle fibre damage. While DMD is characterized by a relatively uniform disease course, disease progression in BMD is more variable.[6-8,19,106] The mechanisms underlying this variability are not fully understood. Factors believed to be involved include the regenerative capacity of muscle tissue, inflammation as well as quantity and quality of the dystrophin protein.[64,70,104,130] Qualitatively, dystrophin has multiple functions that depend on the presence of functional domains within the protein, most importantly the N-terminal actin-binding and the C-terminal beta-dystroglycan-binding domains. While the connecting 'central rod domain' appears to be largely dispensable, the way in which remaining spectrin-like repeats are phased likely impacts dystrophin functionality.[202] An important role for dystrophin quantity has been suggested by previous reports, although there is still uncertainty about the nature of this relationship as both linear relations and a threshold effect have been suggested.[10,19,22,25,56,64,104,106,107,203] The interest in this possible relationship increased with the development of the exon skipping technology in DMD.[170,175] Clinical trials testing systemic delivery of antisense oligonucleotides (AONs) targeting exon 51 have shown restoration of dystrophin levels of up to 18%.[138,139]

In the present study we investigated the relationship between dystrophin levels quantified from a freshly obtained muscle biopsy and disease severity in 33 BMD patients, including 13 patients with an exon 45-47 deletion.

Material and methods

Patients

Adult BMD patients registered in the Dutch Dystrophinopathy Database were eligible to participate, when complying with the following inclusion criteria: (1) an in-frame mutation in the *DMD* gene OR (2) any other mutation in the *DMD* gene AND a mild disease course, defined by age at wheelchair dependence after the age of 16 OR (3) no known mutation in the *DMD* gene AND reduced dystrophin levels in muscle biopsy. Patients using mechanical ventilation were excluded. During a one-day visit quantitative muscle testing of several muscle groups in upper and lower extremity and (optionally) a muscle biopsy of the anterior tibial (TA) muscle was obtained from the study participants. The TA was chosen because it usually contains an adequate amount of muscle tissue for dystrophin analysis in BMD patients with a wide range of disease severity, and since this muscle has also been used in several DMD exon skip trials.[139,140] Additionally, a quantitative MRI of the lower leg was obtained from the subgroup of participants with an exon 45 to 47 deletion who did not have a pacemaker or ICD, or severe contractures. The local medical ethics committee approved the study and written informed consent was obtained from all subjects.

Strength measurements

Quantitative muscle testing (QMT) was used to assess the maximal voluntary isometric contraction (MVIC). Measurements of the following muscle groups were obtained: elbow flexion and extension, handgrip and knee and hip flexion and extension. Ankle dorsiflexion was not included, as measurement of this muscle group has been shown to be unreliable.[201] Measurements were performed as described by Hogrel et al.[201] Wheelchair dependent participants unable to make the transfer to the research bench were examined in their wheelchair. The mean of the bilaterally-tested muscle groups was calculated. To minimize the possible confounding effect of age and weight on the analyses, a z-score for muscle strength of the individual muscle groups was also calculated, taking these factors into account.[201] QMT was also performed in twenty male age-matched healthy volunteers.

Disease milestones

Disease milestones were used to compare disease severity between BMD patients, and thus to partially correct for the wide range of ages. A structured natural history was obtained from the 89 BMD patients with a genetically confirmed in-frame deletion in the Dutch Dystrophinopathy Database, including our participants. The data were used to determine the median ages as well as the 33th and 66th percentile at which the following disease milestones were reached: first motor symptoms, difficulty walking stairs, use of walking aids (braces, cane, walker or wheelchair) and wheelchair dependence.

Western blot analysis

The muscle biopsy from the TA was processed as previously described by Van Deutekom et al.[140] Protein lysates were generated from muscle biopsies and Western blotting was performed according to previously described methods.[140] Monoclonal NCL-DYS1 (dilution 1:100, Novacastra, UK) or polyclonal ab15277 (dilution 1: 200 Abcam, UK) were used to detect dystrophin. Rabbit polyclonal antibody to sarcomeric alpha-actinin (ACTN2) ab72592 (dilution 1: 500 Abcam, UK) was used as a loading control. Blots were visualized and quantified with the Odyssey system and software (Li-Cor, USA) as described previously.[140] Samples obtained from the TA and medial and lateral vastus muscles of five healthy males were used as reference samples. For each patient sample at least 2 technical replicates were performed.

Immunohistochemistry

Sections of 10 µm were cut from the TA biopsies using a Shandon Cryotome (Thermo Fisher Scientific Co., Pittsburgh, PA, USA). Sections were fixed for 1 min with ice-cold acetone. Goat polyclonal dystrophin diluted 1:50 (SC-7461, Santa Cruz Biotechnology, USA) was used to detect dystrophin. Alexa-fluor 488 donkey-anti goat IgG (A11055, Invitrogen, the Netherlands) diluted 1:1000 was used as a secondary antibody. Slides were analysed using a fluorescence microscope (DM RA2; Leica Microsystems Wetzlar, Germany), and digital images were taken using a CCD camera (CTR MIC; Leica Microsystems).

Muscle MRI

In BMD patients with an exon 45-47 deletion MR Images were acquired on a 3T scanner (Achieva, Philips Healthcare, Best, The Netherlands) from the left lower leg using a 14-cm diameter two-element receive coil and body coil excitation. The scanning protocol consisted of a T1-weighted sequence (25 five mm slices, 0.5 mm gap, repetition time (TR) 600 ms, echo time (TE) 16 ms) and a 3-point Dixon sequence (25 five mm slices, 0.5 mm gap, TR 400 ms, first TE 4.41, echo spacing 0.76 ms, flip angle 8°) with multipeak analysis.[204] The mean fat fraction per muscle was calculated from regions of interest (ROIs) using Medical Image Processing, Analysis and Visualization (MIPAV) software. The medial and lateral head of the gastrocnemius, soleus, flexor digitorum longus, flexor hallucis longus, posterior tibialis, extensor hallucis longus, peroneus, tibialis anterior and extensor digitorum longus were analysed.

Statistics

Pearson's correlation test was used to analyse the relationship between dystrophin expression, MVIC score and the mean fat fraction of the muscles. Dystrophin expression levels for the groups based on disease milestones were compared using an independent-samples Student's t-test. Differences between participants and non-participants as well as differences in disease milestones were analysed by Kaplan-Meier survival analysis, using the Log Rank test. SPSS version 16 for Windows (SPSS Inc., Chicago IL) was used. The level of significance was set at $p<0.05$. Values are shown as mean ± SD.

Results

Patients

We approached 60 adult Becker patients to participate in the study of whom 33 consented to participate (Table 1). Reasons not to participate included the physical burden, inability to attend due to work and travel distance to the Leiden University Medical Center. Participants did not differ significantly from non-participants in terms of age or age at reaching disease milestones (Table 2). Mean age of participants was 40 years (± 13, range 19-66 years) versus 44 years (± 13, range 19-65 years) in the non-participating patients. The study population represented a wide clinical variety (Supplementary Table 1), ranging from a patient of 26 years who only noticed mild impairment while walking upstairs carrying heavy tools to

Table 1. Included BMD participants.

	DMD phenotype (loss of ambulation <12 years)	Intermediate phenotype (loss of ambulation 13-16 years)	BMD phenotype (loss of ambulation >16 years
In-frame deletion	1	-	29
Out of frame deletion or point mutation	-	-	2
Unknown mutation, reduced dystrophin levels	-	-	1

Table 2. Characteristics of participating and non-participating BMD patients.

	Study participants n=33		Non participants n=27		p-value
Age	40 (± 13)		44 (± 13)		0.27
	Events	*Estimated mean*	*Events*	*Estimated mean*	
First symptoms	31	12 (± 1.6)	26	11 (± 2.1)	0.41
Walking stairs	26	30 (± 3.0)	25	24 (± 2.7)	0.16
Walking aid	19	41 (± 3.6)	20	36 (± 3.4)	0.49
Wheelchair dependence	8	54 (± 3.2)	11	49 (± 3.4)	0.33

a patient with a DMD-like phenotype (wheelchair-dependence at the age of 10), but with an in-frame deletion in the *DMD* gene and dystrophin expression as assessed by Western blot (see below).

Dystrophin quantification

Fresh muscle biopsies were obtained from 27 patients. Six patients refused a biopsy because of previous negative experiences. Dystrophin expression as assessed by Western blot (Figure 1 and Supplementary Figure 1) ranged from 4% to 71% of normal human skeletal muscle as detected by the rod-domain antibody (NCL-DYS1) and from 3% to 78% using the C-terminus antibody (AB15277). The mutation of one patient (exon 29;c.3940C>T,p.Arg1314X) resulted in the deletion of exon 29 on RNA transcript level, which encodes the epitope of the rod-domain antibody and was therefore only recognized by the C-terminus antibody. As there was a good correlation between the dystrophin levels detected by the two antibodies (R 0·87; p<0·001) further analyses were performed using the C-terminus antibody. Using immune fluorescence analysis we observed homogeneous staining for most patients (16/22), generally at lower intensity than the normal control. For a few patients we observed both dystrophin positive and

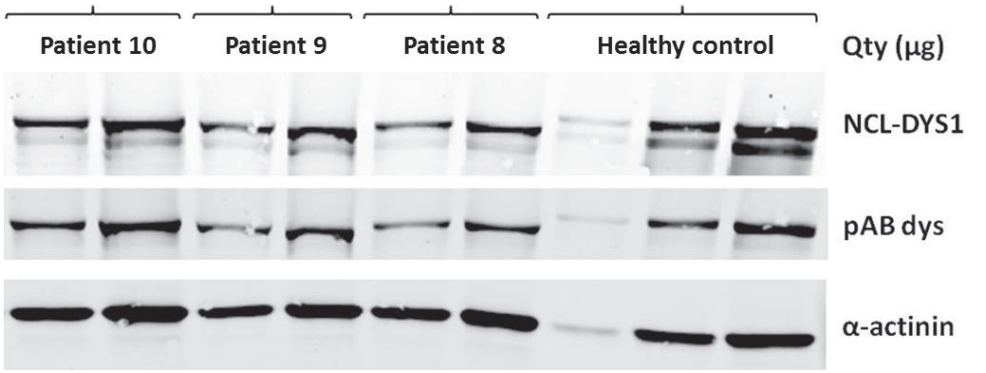

Figure 1. Example of Western blot analysis on total protein extracts from three BMD patients and one healthy control. The rod-domain antibody (NCL-DYS1), C-terminus antibody (pABdys) and loading control (α-actinin) are shown. As anticipated from the deletions, the proteins in the BMD patients are slightly smaller than those in the healthy control. In the BMD group both patients with uniform and mosaic dystrophin expression were found by immunofluorescent staining.

negative fibres (5/22), while for one patient the biopsy was so fibrotic we did not observe any dystrophin staining (see Supplementary Figure 2 for representative examples).

Strength measurements

Quantitative muscle testing of elbow flexion and extension, handgrip and hip flexion and extension was performed in all patients. Due to inability to transfer to the research bench testing of knee flexion and extension was not possible in one and four patients respectively. A significant correlation existed between the MVIC-scores of all seven individually tested muscles. The mean MVIC-sum of the five muscle groups tested in all patients was 99 kg (± 59 kg, 15-186 kg). There was no significant relationship between the MVIC-sum score and the age of patients at the time of QMT-testing (R -0.15; p= 0.41) (Figure 2a). However, when analysing our subgroup of thirteen patients with an exon 45-47 deletion a significant relationship between MVIC-sum score and age was found (R -0.74; p=0.004). The mean MVIC-sum score of age-matched healthy male volunteers (mean age 42 ± 13 years, range 20-62 years) was 182 kg (± 23 kg, 142-220 kg), and showed no significant relationship within this age range (R -0·41; p= 0·07).

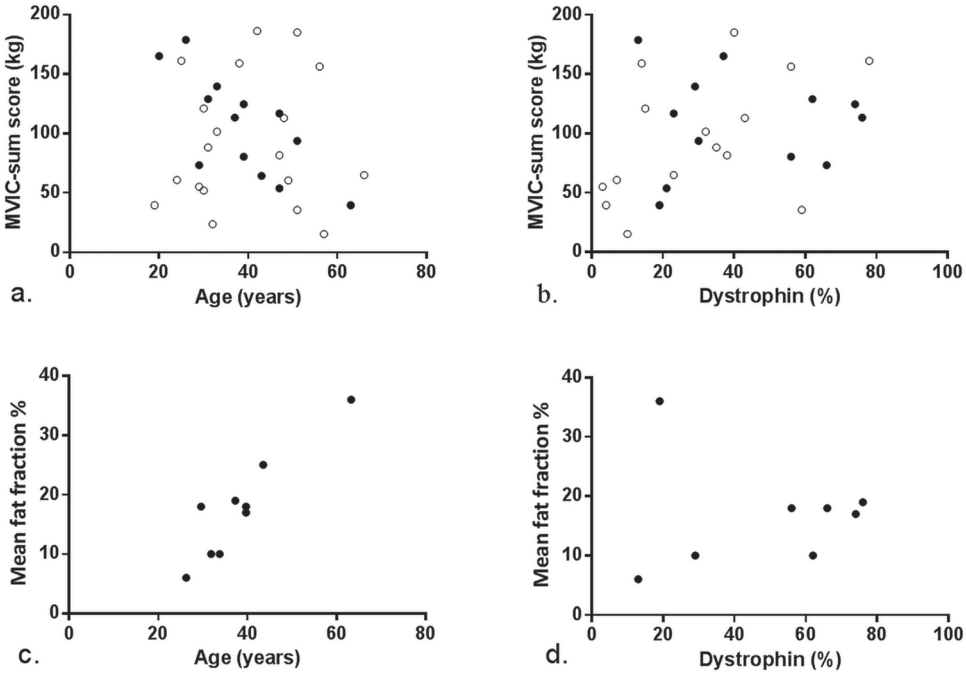

Figure 2. Correlation between age and dystrophin expression with the MVIC-score and the mean fat fraction of the lower leg on MRI.

Data of the subgroup with an exon 45-47 deletion are represented in closed dots, all other mutations in open circles. There is an evident relationship for both the MVIC-score (a.) and the mean fat fraction (c.) with age of the patients in the deletion 45-47 subgroup (R -0.74; p 0.004 and R 0.92; p <0.001 respectively). No relationship is found between dystrophin expression and MVIC-score (b.) or mean fat fraction on MRI (d.).

No relationship was found between dystrophin expression and disease severity, as measured by MVIC-sum score in the whole group (R 0.30; p 0.13) or the exon 45-47 deleted subset (R -0.10; p 0.98) (Figure 2b). This relationship was also absent when analysing all muscle groups individually. To decrease the possible confounding effect of age related differences in muscle strength, the analysis was subsequently performed in three subgroups: patients aged 18-30 (n=8), 31-45 (n=8) and 46 and older (n=11). No significant correlation between dystrophin levels and MVIC-sum score was present in any of these groups (R 0.40 p 0.33; R -0.30 p 0.47 and R 0.46 p 0.16 respectively).

Analyses correcting for both age and weight were added using the z-scores calculated using data from Hogrel, showing again no correlation between dystrophin levels and muscle strength for the whole group (R 0.23 p 0.25) as well as the exon 45-47 subgroup (R -0.71 p 0.83).[201]

Disease milestones

The median age of the disease milestones in 89 BMD patients (aged 3-66 years) was 6 years for 'first motor symptoms', 20 years for 'difficulties walking stairs', 28 years for use of a walking aid and 33 years for wheelchair dependency. For each milestone we grouped our patients with known dystrophin levels based on whether that milestone presented before or after the median age of that milestone (Figure 3a). No significant differences were found between dystrophin levels of the two groups for 'first motor symptoms' (p 0.39), 'difficulties walking stairs' (p 0.91) and 'use of a walking aid' (p 0.25). As too few patients were wheelchair dependant, statistical analysis was not possible for this milestone. When this same analysis was repeated using three instead of two groups, with cut-off points based on the 33rd and

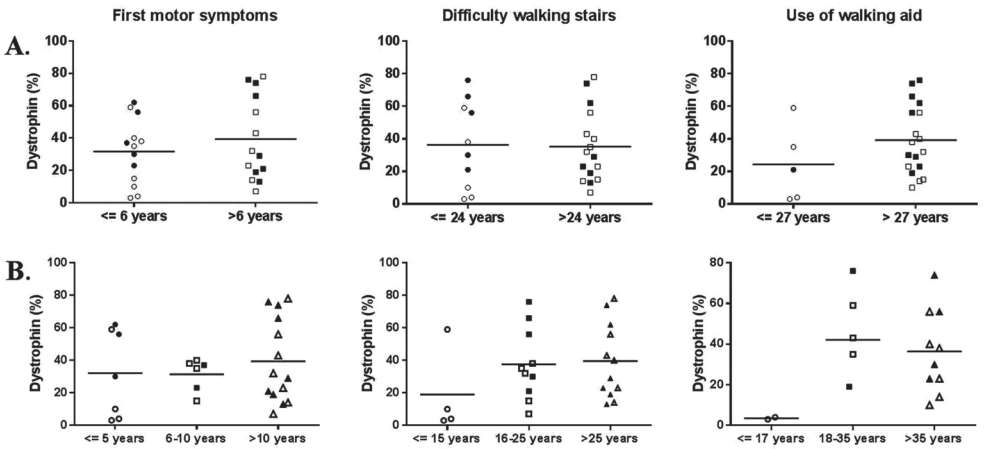

Figure 3. Distribution of dystrophin levels according to age at reaching disease milestones.
Data are shown comparing 89 BMD patients reaching the milestones first motor symptoms, difficulty walking stairs and use of a walking aid. Grouping was performed using the median age (A.) and the 33rd and 66th percentile (B.) at reaching the specific milestone. The horizontal lines mark the average dystrophin level within the groups. Data for the patients with an exon 45-47 deletion are presented in black dots, data for all other patients in open circles. Note that grouping of a specific patients can be different for different milestones.

66th percentile of age at reaching a certain disease milestones, no significant difference in dystrophin levels was present either (Figure 3b).

When analysing the exon 45-47 deletion subgroup the dystrophin levels were not significantly different for two disease milestones (p 0.94 and p 0.40 for first motor symptoms and difficulties walking stairs, respectively). Too few patients had reached the other milestones to include these items in the analysis.

Muscle MRI

An MRI scan was obtained from nine patients with an exon 45-47 deletion. The lower leg showed a distinct pattern of muscle involvement, with the medial head of the gastrocnemius muscle being most severely affected, followed by the lateral head of the gastrocnemius and the peroneus muscle (Figure 4). The TA was relatively spared in all patients, but still showed a significant increase of the fat fraction with age (R 0·94, p<0.001). There was no correlation between dystrophin levels and fatty infiltration on MRI (R -0·03; p 0·95) (Figure 2d). There was however a significant correlation between patients' age and mean fat fraction of the lower leg muscles (R 0·92; p<0·001) (Figure 2c).

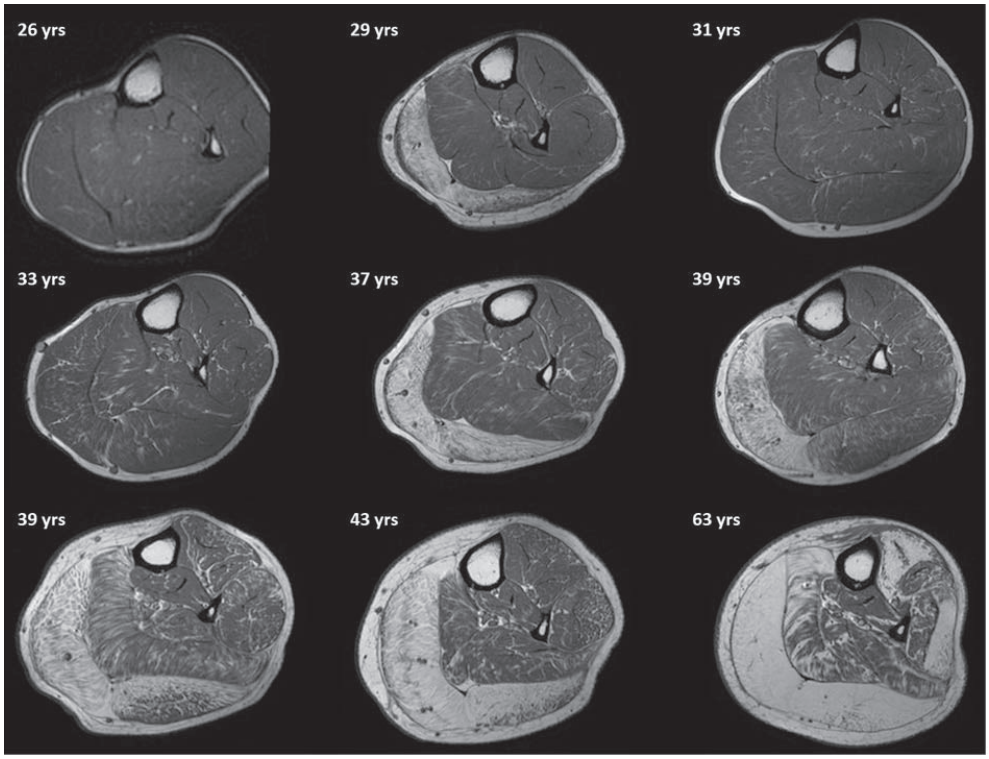

Figure 4. Transversal image of lower leg of BMD patients with an exon 45-47 deletion.
There is an evident increase in the mean fat fraction with age. The changes are most evident for the medial head of the gastrocnemius muscle and in later stages also for the lateral head.

Discussion

We studied a group of BMD patients with a large diversity in disease severity, as well as a wide range of different forms of dystrophin, with 18 different mutations, and assessed dystrophin levels from freshly acquired muscle biopsies, with dystrophin levels ranging from 3 to 78%. In this group of BMD patients there was no linear relationship between dystrophin levels and disease severity, defined by quantitatively measured muscle strength, fatty infiltration on MRI or by clinical milestones.

The relationship between the variable disease severity observed in BMD patients and dystrophin levels has been of increasing interest with the development of exon skipping therapy for DMD patients. Although a small amount of dystrophin in muscle is better than none, it remains unclear what the relationship between dystrophin levels and disease severity entails. Some previous studies found indications that higher dystrophin levels are associated with a milder disease course.[10,19,22,56,104,106] Others reported a threshold effect.[25,107,203] However, these studies pooled data from patients with different mutations. Becker patients with in-frame deletions on the 5'-end of the *DMD* gene generally have a more severe disease course, including an earlier age of cardiac involvement.[22,25,166,205] Therefore, it is likely that the pooling of different mutations has influenced these results.

A recent study by Anthony et al. focused on a more selected group of patients with a mutation bordering exon 51 or exon 53 and patients with an exon 45-55 deletion, therefore limiting the effect of the location of the mutation on disease progression to some extent.[104] Their results suggested that higher dystrophin levels were associated with a less severe disease course. However, their analysis was performed in asymptomatic and mild patients with relatively high dystrophin levels (all above 50%). Therefore, the results cannot be extrapolated to the entire BMD disease spectrum. In our subgroup of 13 patients with an exon 45-47 deletion no relationship between dystrophin levels (varying from 13-76%) and disease severity was found. In contrast, in this subgroup there was an evident relationship between disease severity and age of the patients. These findings are supported by the MRI data, showing a progressive increase in fatty infiltration with age. We believe this finding illustrates that the study of subsets of BMD patients with the same mutations can identify relations otherwise obscured.

Although we found no relationship between dystrophin levels and disease severity, our four patients with dystrophin levels below 10% showed low MVIC-sum scores and early onset of symptoms (Figure 3 and Supplementary Table 1). This finding implies a threshold effect, which is conform several previous clinical studies suggesting that dystrophin levels below 10% are indicative of a more severe disease course.[25,107,203] This could suggest that a relatively small amount of dystrophin is sufficient to result in a disease course that is milder than DMD, but relatively more severe than 'typical BMD'. This is also in accordance with previous reports of several mouse models, showing threshold levels between 15-20%, and with a recent study in mice showing that in a dystrophin and utrophin negative background, very low levels of dystrophin (up to 5%) improved survival, but not histology, levels between 5 and 15% further improved survival and fibrosis and levels of over 15% normalized survival and further improved fibrosis.[206-209] The clinical disease course in patients with an exon 45-47 deletion was quite uniform, but

still there was variability, suggesting a role for additional factors such as differences in regenerative capacity, genetic modifiers or inflammation.

Exons 45-47 encode the last half of spectrin-like repeat 17 as well as repeat 18 of the dystrophin protein. It is known that repeat 16 and 17 (encoded by exon 42-45) are involved in nNOS-binding.[117,210] Anthony et al. and Gentil et al. both have shown a relationship between (properly located) nNOS levels and disease severity.[104,123] Possibly, differences in nNOS expression could be one of the factors involved in the disease variability of the exon 45-47 deleted group.

As dystrophin plays an important role in muscle fiber membrane stability any change in protein structure could lead to a decreased functioning. The findings that in-frame mutations at the 5'-end of the *DMD* gene lead to a more severe BMD or a DMD phenotype suggest that this domain is less dispensable for protein function. In contrast the central rod domain that connects the N-terminal actin-binding and C-terminal beta-dystroglycan binding domains allows more flexibility, as is e.g. underlined by the finding that a deletion of >60% of the rod domain (deletion exon 17-48) resulted in a very mild BMD phenotype.[68] Structural changes of the dystrophin protein, caused by a specific mutation, will impair dystrophin function to an extent, characteristic for that mutation.

Our study suggests a direct effect of the exact mutation on the clinical phenotype of BMD patients. This finding has important implications for future BMD studies, where it will be essential to account for different mutations when looking at other possible contributing factors to disease severity. The possible disease modifying effect of dystrophin quality also has implications for exon skipping trials. As this therapy aims to change a DMD phenotype into a BMD phenotype, knowledge of the specific BMD phenotypes is important, as for some mutations ameliorating of the phenotype may be more beneficial than for others.

The suggestion of a threshold effect in this study could benefit therapeutic studies aiming to restore dystrophin in DMD patients. Clinical trials testing systemic delivery of antisense oligonucleotides (AONs) targeting exon 51 have shown dystrophin levels of up to 18%.[138,139] If relatively low levels of dystrophin are sufficient to result in a relatively mild BMD phenotype, it may be expected that patients may improve with these therapies.

There are several limitations to our study. Firstly, several other studies used muscle biopsies from the quadriceps muscle, while we chose to biopsy the anterior tibial muscle, as it contains viable muscle tissue over wider clinical severity. Variation in dystrophin expression between muscles cannot be ruled out, but there are no indications that this could limit the interpretation. A previous immunohistochemical study reported no significant variation in dystrophin levels between various muscles in DMD patients, nor between biceps brachialis and lateral vastus muscles in a group of BMD patients.[22,211] Additionally, no significant variation in dystrophin expression was found in different skeletal muscles in mice.[212] Although our study is the largest study in BMD patients comparing disease course and dystrophin levels using fresh biopsies, our study could be underpowered to detect a possible relationship between dystrophin levels and disease severity. Larger subgroups of BMD patients with the same mutation would be necessary to study such a relationship, which, due to the rareness of BMD, will be challenging. However, in

our subgroup of patients with an exon 45-47 deletion we did find a relationship with age, but not with dystrophin. Thirdly, although there is no significant difference in disease course between participants and non-participants, a small trend favoring the participation of milder patients appears to be present. This is probably caused by the greater burden on severely affected patients to participate.
In summary, our study shows that there is no linear correlation between dystrophin levels and disease severity in BMD patients and that disease progression is uniform in a subgroup of BMD patients with an exon 45-47 deletion, as demonstrated by the decrease of muscle strength as well as increase of fatty infiltration of muscle. In contrast to the whole BMD group, these phenomena increase with age at a constant pace in the exon 45-47 deletion group, indicating that the mutation is likely an important factor in determining disease severity. Furthermore, there are indications for a threshold rather than a dose-effect relationship and the fact that this threshold could likely be around 10% of normal levels (at least for the exon 45-47 deletion) has important implications for therapeutic approaches aiming at dystrophin restoration.

Acknowledgements

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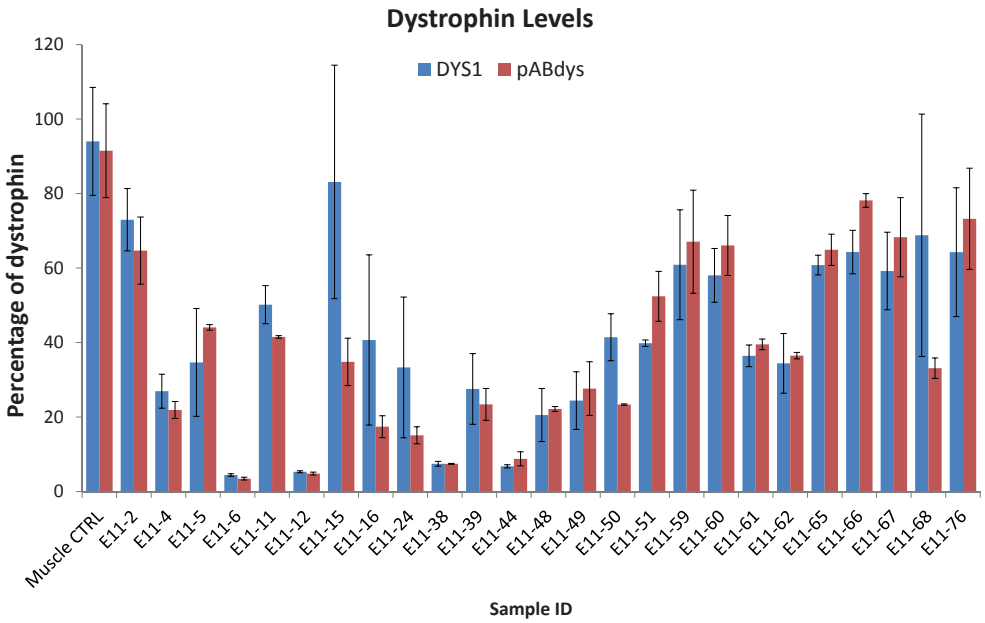
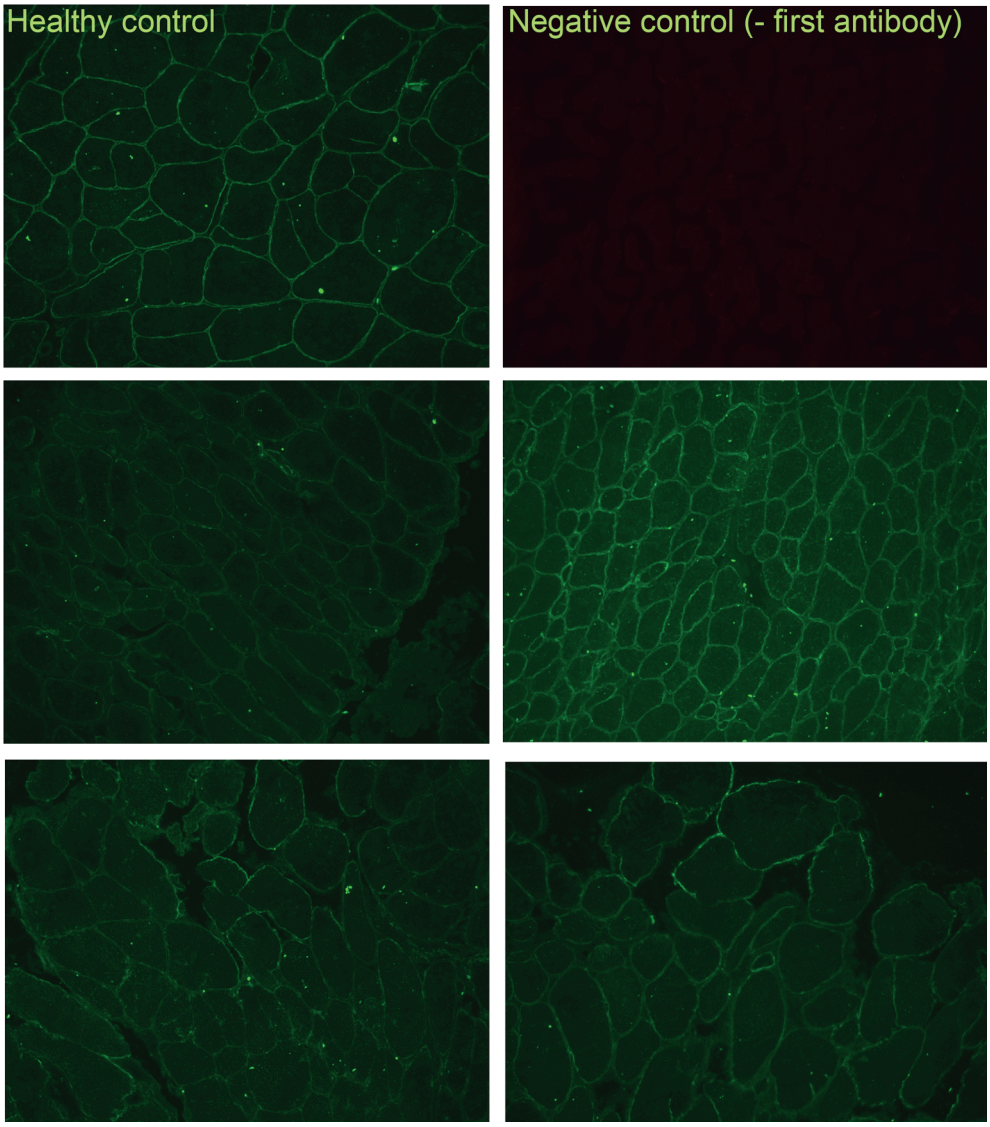
Supplementary Table 1. Overview of data and clinical characteristics of study participants.

Patient	Mutation	Age at examination (years)	Biopsy	MRI	QMT sum score (kg)	Dystrophin (%)	Disease Milestones (years)			
							First Symptoms	*Walking Stairs*	*Walking Aid*	*Wheelchair Dependence*
1	del 45-47	47	Yes	No	117	23	6	38	43	-
2	del 45-47	51	Yes	No	94	30	5	18	51	-
3	del 45-47	26	Yes	Yes	179	13	21	-	-	-
4	del 45-47	63	Yes	Yes	54	19	25	30	35	60
5	del 45-47	47	Yes	No	54	21	17	19	25	37
6	del 45-47	39	Yes	Yes	80	56	5	20	38	-
7[1]	del 45-47	37	Yes	Yes	113	76	16	20	30	-
8	del 45-47	20	Yes	No	165	37	6	-	-	-
9	del 45-47	29	Yes	Yes	73	66	12	20	-	-
10	del 45-47	39	Yes	Yes	125	74	21	35	-	-
11[1]	del 45-47	33	Yes	Yes	140	29	25	28	-	-
12	del 45-47	43	No	Yes	64	NA	6	35	35	-
13	del 45-47	31	Yes	Yes	129	62	2	-	-	-
14	del 03-04	31	Yes	No	88	35	6	25	25	-
15	del 03-05	33	Yes	No	102	32	18	25	-	-
16	del 03-07	57	Yes	No	15	10	4	12	40	45
17	del 05	19	Yes	No	40	4	2	4	4	10
18	del 10-22	47	Yes	No	82	38	6	20	37	-
19	del 10-30	30	No	No	52	NA	4	4	20	-
20	dup 14-42	66	Yes	No	65	23	18	60	65	-
21	Exon 19: c.2380+3A>C	29	Yes	No	55	3	2	4	9	25
22	Exon 26: c.3515G>A; p.Trp1172X	30	Yes	No	121	15	6	25	-	-
23	Exon 29: c.3940C>T;p. Arg1314X	38	Yes	No	159	14	-	-	-	-
24	del 45-55	51	Yes	No	185	40	6	-	-	-
25	del 45-49	32	No	No	24	NA	8	18	20	27
26	del 48-49	42	No	No	186	NA	11	-	-	-
27	del 30-44	24	Yes	No	61	7	12	21	-	-
28	del 45-48	49	No	No	60	NA	12	30	25	-
29	del 45-48	48	Yes	No	113	43	15	30	35	49
30	del 45-48	51	Yes	No	36	59	4	13	25	39
31	del 48-49	25	Yes	No	161	78	-	-	-	-
32	Unknown	56	Yes	No	156	56	18	50	-	-
33	del 45-55	63	No	No	124	NA	6	43	63	-

NA: data not available, hyphen in section: patient has not reached the specific milestone.
[1] brothers



Supplementary Figure 1. Average dystrophin levels assessed by Western blot for BMD patients.
Protein was isolated from muscle biopsies and dystrophin levels were quantified by quantitative Western blotting using the Odyssey system setting dystrophin levels observed in healthy muscles to 100% and using alpha-actinin to correct for equal loading. Each sample was measured at least two times with a monoclonal (DYS1) and a polyclonal (pABdys) antibody. Average dystrophin levels are shown. Error bars show the standard deviation.



Supplementary Figure 2. Immunofluorescent staining for dystrophin.
Dystrophin staining is continuous and bright for the healthy control (top left panel). Representative images of cross sections from BMD patients are shown, showing either continuous staining at a lesser intensity (middle panels) or a mosaic pattern (bottom panels).