Dystrophin quantification and clinical correlations in Becker muscular dystrophy: implications for clinical trials

Karen Anthony1*, Sebahattin Cirak1*, Silvia Torelli1, Giorgio Tasca2, Lucy Feng1, Virginia Arechavala-Gomeza1, Annarita Armaroli3, Michela Guglieri4, Chiara Straathof6, Jan Verschuuren5, Annemieke Aartsma-Rus6, Paula Helderman-van den Enden6, Katherine Bushby4, Volker Straub4, Caroline Sewry1, Alessandra Ferlini3, Enzo Ricci7, Jennifer Morgan1, Francesco Muntoni1.

* These two authors have equally contributed to the manuscript.

1 The Dubowitz Neuromuscular Centre, UCL, Institute of Child Health, London, UK. 2Don Carlo Gnocchi Onlus Foundation, Italy. 3Section of Medical Genetics, Department of Experimental and Diagnostic Medicine, University of Ferrara, Ferrara, Italy. 4Institute of Genetic Medicine, Newcastle University, Newcastle, UK. 5Department of Neurology, Leiden University Medical Centre, Leiden, the Netherlands. 6Department of Human Genetics, Leiden University Medical Centre, Leiden, the Netherlands. 7Institute of Neurology, Catholic University, Rome, Italy.

Correspondence should be addressed to:
Professor Francesco Muntoni
Dubowitz Neuromuscular Centre, UCL Institute of Child Health,
30 Guilford Street, London, WC1N 1EH, UK.
Telephone: +44 20 7905 2111. Fax: +44 20 7905 2832.
E-mail: f.muntoni@ich.ucl.ac.uk
Abstract

Duchenne muscular dystrophy (DMD) is caused by mutations in the DMD gene which disrupt the open reading frame and prevent the full translation of its protein product, dystrophin. Restoration of the open reading frame and dystrophin production can be achieved by exon skipping using antisense oligonucleotides targeted to splicing elements. This approach aims to transform the DMD phenotype to that of the milder disorder, Becker muscular dystrophy (BMD), typically caused by in-frame dystrophin deletions that allow the production of an internally deleted but partially functional dystrophin. There is ongoing debate regarding the functional properties of the different internally deleted dystrophins produced by exon skipping for different mutations; more insight would be valuable to improve and better predict the outcome of exon skipping clinical trials. To this end we have characterised the clinical phenotype of 17 BMD patients harbouring in-frame deletions relevant to on-going or planned exon skipping clinical trials for DMD and correlated it to the levels of dystrophin, and dystrophin-associated protein expression. The cohort of 17, selected exclusively on the basis of their genotype, included four asymptomatic, 12 mild and one severe patient. All patients had dystrophin levels of more than 40% of control and we observed significantly higher dystrophin (p = 0.013), β-dystroglycan (p = 0.025) and neuronal nitric oxide synthase (nNOS, p = 0.034) expression in asymptomatic individuals versus symptomatic BMD patients. Furthermore, grouping the patients by deletion, BMD patients with deletions with an endpoint of exon 51 (the skipping of which could rescue the largest group of DMD deletions) showed significantly higher dystrophin levels (p = 0.034) than those with deletions ending with exon 53. This is the first quantitative study on both dystrophin and dystrophin-associated protein expression in BMD patients with deletions relevant for on-going
exon skipping trials in DMD. Taken together our results indicate that all varieties of internally deleted dystrophin assessed in this study have the functional capability to provide a substantial clinical benefit to DMD patients.
Keywords

Becker muscular dystrophy
Duchenne muscular dystrophy
nNOS
Dystrophin-associated glycoprotein complex
Therapy

Total number of words

Total number of words in the text (excluding references, tables and figure legends) = 5712 words.
Dystrophin quantification in BMD

**Introduction**

Duchenne muscular dystrophy (DMD) is a fatal X-linked neuromuscular disorder caused by mutations in the *DMD* gene that disrupt the open reading frame and prevent the full translation of its protein product, dystrophin. The majority of *DMD* gene mutations are deletions (~65%) although duplications (~10%), small mutations (~22%) and deep intronic mutations (~2-3%) are also documented (Muntoni *et al.*, 2003, Abbs *et al.*, 2010). Patients develop muscle weakness in the early years of life and lose the ability to walk by their early teens; unless appropriate respiratory and cardiac treatment is initiated, affected individuals typically die before reaching their twenties.

Dystrophin is localised to the inner part of the sarcolemma of muscle fibres where it is associated with other proteins as part of the dystrophin-associated protein complex (DAPC). Dystrophin plays an important role in stabilising the muscle fibre against the mechanical forces of muscle contraction by providing a shock-absorbing connection between the cytoskeleton and the extracellular matrix and is also believed to have a role in signalling. The absence of dystrophin is thought to render muscle cells susceptible to stretch-induced damage and necrosis. Dystrophin consists of four main functional units; the N-terminus contains two calponin homology domains responsible for actin-binding. The central rod domain consists of 24 spectrin-like repeats with four interspersing hinge domains which also contribute to actin binding (Ervasti, 2007, Le Rumeur *et al.*, 2010). The cysteine rich and the C-terminal domains are required for binding to β-dystroglycan (BDG), a crucial protein of the dystroglycan complex which helps maintain the structural integrity of muscle tissue (Ervasti, 2007, Le Rumeur *et al.*, 2010). In addition to a major structural role, a specific domain of dystrophin (exons 42 – 45 encoding spectrin-like repeats 16 and 17
within the rod domain) is necessary for the sarcolemmal localisation of neuronal nitric oxide synthase (nNOS) (Lai et al., 2009) which contributes to the fine-tuning of muscle blood flow during physical activity. In DMD muscle, nNOS is absent from the sarcolemma leading to paradoxical exercise-induced vasoconstriction which contributes to the ongoing muscle damage.

A clinically milder allelic disorder, Becker muscular dystrophy (BMD), is caused by in-frame dystrophin mutations, typically involving part of the central rod domain, that preserve the reading frame permitting the translation of an internally deleted dystrophin protein. There is a large variation in the clinical severity of BMD patients with some individuals able to experience a near normal lifestyle and lifespan while others lose the ability to walk in their late teens or early twenties (Bushby et al., 1993). Although the reasons for this variability are not fully understood, it is likely that both the functionality and levels of the internally deleted dystrophin proteins play a significant role.

Naturally occurring alternative splicing of dystrophin pre-mRNA can restore the reading frame in DMD as a result of the skipping of some exons and is thought to explain the presence of dystrophin positive ‘revertant fibres’. This phenomenon has been well characterised in the mdx mouse (Lu et al., 2000), and in DMD (Klein et al., 1992, Arechavala-Gomeza et al., 2010). There has been debate regarding the functionality of both the dystrophin in revertant fibres and the internally deleted dystrophin of BMD. The milder BMD phenotype and the fact that the internally deleted proteins are correctly localised to the sarcolemma and accompanied by the DAPC suggests that they are indeed functionally competent (Matsumura et al., 1994).
There is currently no effective treatment for DMD; however a promising therapeutic strategy is to transform the DMD phenotype into the milder BMD phenotype through the restoration of the open reading frame and the production of internally deleted, BMD-like dystrophin molecules. This can be achieved by exon skipping using oligonucleotides targeted to splicing elements (splice switching oligonucleotides (SSO)) (Wood et al., 2010). We and others have previously demonstrated the local restoration of dystrophin expression in DMD patients using either morpholino SSO AVI-4658 or the 2’-O-methyl phosphorothioate PRO-051 which induce the skipping of exon 51 in dystrophin mRNA (Kinali et al., 2009, van Deutekom et al., 2007). Skipping of exon 51 could rescue the largest group (13%) of DMD mutations (Aartsma-Rus et al., 2009) and the first studies aimed at assessing the biochemical efficacy and clinical safety of systemically administered SSOs are now emerging (Goemans et al., 2011, Cirak et al., 2011)

Multi-exon skipping is an attractive alternative to the skipping of single exons and has the potential to rescue over 50% of all DMD deletions. According to bioinformatical analysis, exons 45 – 55 are the most optimal set of exons to skip for conversion of DMD to BMD (Beroud et al., 2007). BMD patients harbouring this deletion can have a mild phenotype, often associated with a late-onset (Ferreiro et al., 2009, Tselikas et al., 2010). There are in fact several reports describing asymptomatic BMD with a variety of deletions, including some with an endpoint in exons 51 or 53 (Ferreiro et al., 2009, Lesca et al., 2007, Melis et al., 1998, Morrone et al., 1997, Muntoni et al., 1997, Saengpatrachai et al., 2006, Sanchez-Arjona et al., 2005, Torelli et al., 2004).

Very few thorough quantitative studies of dystrophin expression and functionality have been performed in BMD patients (Bushby et al., 1993, Beggs et al., 1991,
Comi et al., 1994, Nicholson et al., 1993, Morandi et al., 1995) and none of these studies used quantitative immunohistochemistry or had sufficient patients with deletions relevant for current exon skipping trial efforts. Several questions remain unanswered, in particular it is not clear which is the optimal target exon(s) for removal and whether different deletions, which could all respond to the skipping of the same exon, are associated with a similar phenotype or not. To this end, we have quantitated dystrophin and several members of the DAPC in skeletal muscle biopsies from 17 BMD patients who harbour in-frame deletions relevant to ongoing exon skipping pre-clinical and clinical trials for DMD. We investigate the association between the clinical severity of BMD with the amount of dystrophin and DAPC proteins in order to better understand and assess both the level and functional properties of the dystrophin produced by exon skipping in future clinical trials.
**Materials and methods**

**Patients**

We performed a multi-centre retrospective study of clinical data from five centres: London, U.K (Dubowitz Neuromuscular Centre at the Institute of Child Health & Great Ormond Street Hospital for Children); Rome, Italy (Institute of Neurology, Catholic University School of Medicine); Ferrara, Italy (University of Ferrara); Leiden, Holland (Leiden University Medical Centre) and Newcastle, U.K (Institute of Genetic Medicine, Newcastle University). A total of 17 patients were selected from a cohort with comprehensive DNA testing using the following inclusion criteria: (1) a confirmed in-frame exon deletion in the dystrophin gene by multiplex ligation-dependent probe amplification (MLPA) and (2) a deletion applicable to exon 51, 53 or 45-55 multi-exon skipping models in DMD. A standardised questionnaire was distributed to obtain information on gene mutation, age of onset, family history, walking ability and specific symptoms such as muscle cramps and myoglobinuria. BMD patient 2 has been described clinically as case A1 in a previous study (Helderman-van den Enden et al., 2010), but protein quantification was not previously studied.

Skeletal muscle biopsies were obtained with informed consent from all 17 BMD patients along with non-myopathic controls (n = 2) and DMD patients (n = 3) for comparative analysis. Muscle biopsies were taken from the quadriceps (12 cases), deltoid (2 cases) or tibialis anterior (3 cases) muscles as indicated in table 1. Control and DMD muscle biopsies (quadriceps) were obtained from the Biobank of the MRC Centre for Neuromuscular Diseases, London U.K.

**Immunohistochemistry**
Muscle biopsies were taken using standard techniques and mounted in OCT then frozen by immersion in isopentane cooled in liquid nitrogen. Immunohistochemistry and subsequent quantitative analysis of all samples was performed as described previously (Arechavala-Gomeza et al., 2010) at the same centre (London) to minimise variability. Primary monoclonal antibodies used were MANDYS106 (MDA monoclonal antibody resource, Prof. Glenn Morris, Oswestry, UK, 1:100), Dys2 (Novacastra, UK, 1:20), α-sarcoglycan (Novacastra, UK, 1:50), β-dystroglycan (Novacastra, UK, 1:20) and β-spectrin (Novacastra, UK, 1:20). A rabbit polyclonal antibody was used to detect n-NOS (Santa Cruz Biotechnology, USA, 1:50). Sections were evaluated using a Leica DMR microscope interfaced to MetaMorph (Molecular Devices, USA) and intensity measurements performed as previously described (Arechavala-Gomeza et al., 2010). The Mann-Whitney test was used for statistical analysis, significance was set at p = 0.05. (* p<0.05 ** p<0.01 *** p<0.001).

**Western blotting**

Western blot analysis was performed at the same centre (London) to minimise variability. Proteins were homogenised from either snap frozen muscle tissue or cryostat cut sections in SDS lysis buffer containing protease inhibitors. For snap frozen muscle samples, 40 µg of total protein was loaded; sections were lysed in a total volume of 50 µl of lysis buffer and 11 µl was loaded. Western blotting was performed with NuPAGE® Novex Tris-Acetate 3-8 % gels (Invitrogen, USA) and PVDF membranes. Membranes were incubated overnight at 4°C with Dys2 (1: 50, Novocastra, UK) and sarcomeric α-actinin (as a loading control, 1:10000, Sigma, UK) primary antibodies. After washing, membranes were incubated with biotinylated anti-mouse IgG (Amersham GE Healthcare, UK) for 1 h followed by horseradish
peroxidase-conjugated streptavidin (Dako, UK) for 1 h. Membranes were visualised using chemiluminescence (ECL Plus, Amersham GE Healthcare, UK) and a Typhoon scanner (Amersham GE healthcare UK). For quantification, dystrophin intensity was normalised to α-actinin using Image J software and expressed as percent of control.
Results
To avoid any potential bias, patients were selected exclusively by their genotype and grouped according to corresponding exon skipping models for DMD. Individual patient’s deletions and their groupings are listed in table 1. Of the 17 patients, eight have deletions ending with exon 51 (model 51) whose skipping would rescue 13% of all DMD mutations (Aartsma-Rus et al., 2009). Four patients have a large deletion of exons 45 – 55 (model MS), multi-exon skipping of these exons in DMD could rescue up to 63% of DMD deletions (Beroud et al., 2007). Finally, five BMD patients have deletions ending with exon 53 (model 53) whose skipping would account for 7.7% of all DMD mutations (Aartsma-Rus et al., 2009).

Clinical characteristics
All the patients in our study had a clinical diagnosis of BMD. According to their skeletal muscle phenotype, the 17 patients were classified as asymptomatic, mild or severe (Table 1). Asymptomatic BMD patients were individuals with no detectable muscle weakness and in whom the only pathological feature was the isolated elevation of serum creatine kinase (CK). Mild BMD was defined by evidence of mild proximal muscle weakness in patients in whom autonomous ambulation and running ability was retained; individuals with more severe weakness and in whom running abilities had either never been acquired or were lost by the end of adolescence were classified as severe. Of the 17 individuals studied, 12 were classified as mild, four asymptomatic and one severe. All patients were ambulant at the time of study with the exception of patient 10 who lost independent ambulation following a femur fracture at the age of 73.
The average age of the 17 patients at the time of study was 25 years and ranged between 10 and 76 years; the average age of onset was 10 and ranged between 2 and 55 years, although this information could not be precisely established in several patients (patients 3, 6, 8, 14, 16 and 17). Seven patients were below the age of 20 at the time of the study, six of whom were classified as mild BMD; the four asymptomatic patients were 13, 23, 25 and 34 years of age at the time of study and the severe patient (number 14) was 25 years old. Thus the differences in clinical severity do not merely reflect differences in age although an influence of age cannot be fully discounted. The patient classified as severe BMD (patient 14, del 45 - 53) had proximal weakness with a Gowers’ time of 3 seconds, a positive Trendelenburg sign and an inability to run.

Cramps were reported in six out of the 17 patients and recurrent myoglobinuria was described in one case. Overall, the clinical severity was heterogeneous within and between the three groups of deletions; patients in the model 51 group were either asymptomatic (3 patients), or mildly affected (5 patients). In the model 53 group three patients were classified as mild; one asymptomatic and one severe; all four patients in the model MS group were classified as mild. Within our cohort, only patient 6 (aged 25 years, 48-51 deletion) suffered from mild dilated cardiomyopathy (DCM) at the time of the study, detected upon routine surveillance.

**Quantification of dystrophin expression**

Dystrophin expression was assessed in all 17 BMD patients by immunohistochemistry using both MANDYS106 (exon 43) and Dys2 (last 17 C-terminal amino acids) dystrophin antibodies and semi-quantitative analysis was performed as previously described (Figs. 1 and 2) (Arechavala-Gomeza et al., 2010). For each group, a DMD
patient with an equivalent out of frame deletion was included for comparative analysis; as expected dystrophin was severely reduced in these three DMD patients, with levels ranging between 4 – 9 % of control. We observed reduced dystrophin staining at the sarcolemma (Fig. 2) which has been reported as a pathological feature of BMD (Beggs et al., 1991, Muntoni et al., 1993). Dystrophin levels across all 17 BMD patients were variable ranging from ~50 % to ~100 % of control as determined by semi-quantitative analysis. In the model 51 group, which includes three patients with a 45 - 51 deletion and five patients with a deletion of exons 48 - 51, the mean dystrophin expression was ~80% of control with both MANDYS106 (range: 48 – 104 %) and Dys2 antibodies (range: 66 – 112 %). The mean level of dystrophin in the model MS group was ~70 % (MANDYS106 range: 62 – 87 %, Dys2 range: 59 - 85 %) which decreased to ~56 % in the model 53 group (MANDYS106 range: 0 – 69 %, Dys2 range: 44 - 89 %). The difference in dystrophin levels between the model 51 and model 53 groups was significant when tested using the MANDYS106 antibody (p = 0.034). Intragroup variability was lowest within the model MS group, perhaps reflecting the fact that this group contains four patients harbouring the same deletion (exons 45 - 55). It is interesting to note that the clinically most severe patient, BMD 14 in the model 53 group, had the lowest dystrophin expression with the Dys2 antibody and also one of the lowest with MANDYS106 (Dys2: 44%, MANDYS106: 57%). On the contrary, the four asymptomatic patients belonging to the model 51 and 53 groups have comparatively higher dystrophin levels with both antibodies. This is illustrated in figure 5 where the difference in dystrophin expression between asymptomatic and mild BMD patients is significant with the Dys2 antibody (p = 0.013). Statistical analysis was restricted to asymptomatic and mild patients as only one patient in our cohort was classified as severe.
The dystrophin quantities detected by MANDYS106 and Dys2 antibodies do not always perfectly correlate. For example, the deletion of BMD patient 13 includes the MANDYS106 epitope in exon 43 and therefore zero dystrophin was detected with this antibody, confirming the high specificity of the MANDYS106 antibody (this data point was excluded from statistical analysis as the zero value does not fairly represent the dystrophin content in this patient). Only one patient, BMD patient 4 (deletion 48-51), showed an unexpectedly large difference between the two antibodies. This individual’s dystrophin level was 48% of control with MANDYS106 and 83% with Dys2.

To confirm the immunohistochemical findings, we next analysed dystrophin expression by western blotting with the Dys2 antibody in two BMD patients from each group (Fig. 3A). Dystrophin bands were detected for all patients at the expected molecular mass, with the patients from the MS model having the lowest molecular mass owing to their larger deletions. Semi-quantitative analysis revealed that model 51 BMD patients had a mean dystrophin expression of 65% (SD ± 2.1) of control, model MS and model 53 had 83% (SD ± 31.8) and 77% (SD ± 19.8) respectively (Fig. 3B). Although a robust comparison between dystrophin quantification methods was not the aim of this study, in most instances we observed comparable expression levels between the two techniques (Fig. 3B). However, for model 51, the dystrophin expression level is substantially lower for BMD patient 3 when calculated by western blotting, possibly reflecting variability in muscle preservation in different blocks of the muscle biopsy studied with the different techniques. Nonetheless, by two independent methods we have demonstrated that our cohorts of asymptomatic or mild BMD patients have dystrophin levels of at least 40% of control.
Expression of proteins of the DAPC

To further assess the functional properties of the BMD dystrophin proteins and to correlate this to clinical severity, we quantified the expression of ASG, BDG and nNOS in the 17 BMD patients by immunohistochemistry (Figs. 2 and 4). ASG and BDG expression was reduced in all BMD patients in comparison to control, and in several cases the level was comparable to that observed in DMD patients. ASG expression varied from 27% to 78% of control whilst BDG expression varied from 37% to 82% of control. Expression of both ASG and BDG was lower in patients belonging to the model MS and model 53 groups; interestingly dystrophin levels determined by immunohistochemistry were also lower in these groups compared to the model 51 group. Higher levels of BDG expression were associated with asymptomatic skeletal muscle phenotypes (asymptomatic vs. mild, $p = 0.025$) whilst no association was found with ASG expression levels (Fig. 5). Clinically severe patient 14 had 64% ASG levels but only 38% BDG whilst mild BMD patient 10 had the highest ASG and BDG levels (70% and 66% respectively) within the model MS group and also the highest dystrophin levels of the group (MANDYS106: 86% and Dys2: 85% of control). Thus in this cohort of patients, BDG expression was significantly associated with mild clinical severity whilst ASG expression was not.

Of the DAPC proteins studied, nNOS showed the most variable expression across all patients (between 16% and 139% of control). The dystrophin nNOS-binding domain is encoded by exons 42 – 45 and so the presence of nNOS at the sarcolemma of BMD muscle is expected to be significantly influenced by the patient’s deletion. Indeed, we demonstrate that BMD patients with disrupted dystrophin nNOS-binding domains (11 out of 17 patients) have lower sarcolemmal nNOS expression despite relatively high levels (>50% of control) of dystrophin (Figs. 4 and 5). This is particularly noticeable...
with the model MS group in which all patients have incomplete nNOS-binding domains and the nNOS levels for all four patients were below 25% of control. Within the model 53 group, asymptomatic patient 17 with an intact nNOS-binding domain has the highest nNOS expression of the whole cohort (102% of control); similarly BMD patients 5, 6 and 8 have the highest nNOS expression within the model 51 group, patients 6 and 8 being classified as asymptomatic. The effect of dystrophin nNOS-binding domain integrity on nNOS expression is further illustrated by the large SD between the sample means of the model 51 group which contains five patients with a complete nNOS-binding domain and three with incomplete binding domains. The clinically most severe patient (BMD 14) has a low nNOS expression, 45% of control; this clinical association is illustrated in figure 5, where the difference in nNOS expression between asymptomatic and mild patients is significant (p = 0.034). As indicated by the coloured circles in figure 5, three out of the four asymptomatic patients have complete nNOS-binding domains and mild BMD patients with complete nNOS-binding domains have a relatively higher nNOS expression level than those with incomplete nNOS-binding domains. Mild BMD patients 4 and 7 (del 48 – 51) with complete binding domains have lower than expected nNOS levels suggesting that an intact dystrophin nNOS-binding domain might not be the only variable to determine sarcolemmal nNOS expression, as recently suggested by others (Finanger Hedderick et al., 2011, Miyagoe-Suzuki and Takeda, 2001). Of the six patients who suffered frequent cramps, three had a complete nNOS-binding domain, thus at least in our cohort the occurrence of cramps does not necessarily depend on the integrity of the nNOS-binding domain.
**Discussion**

We have quantitatively assessed the levels of dystrophin and dystrophin-associated protein expression in 17 BMD patients grouped by deletion according to currently studied exon skipping models for DMD. Using two independent methods we demonstrate that all the BMD patients in our cohort have reduced dystrophin expression levels of at least 40% of control. A previous study of X-linked dilated cardiomyopathy (XLDCM) families determined that dystrophin levels of 30% of control can be sufficient to avoid muscle weakness in these families, although these patients expressed lower levels of normal dystrophin (Neri *et al.*, 2007). Thus our data indicate that the production of internally deleted dystrophin proteins by exon skipping should provide a substantial benefit to DMD patients.

Overall, patients in the model 51 group had higher dystrophin and DAPC levels than patients in the other two groups with significant differences in dystrophin and nNOS expression. This lends further encouragement to the ongoing exon 51 skipping clinical trials for DMD (Kinali *et al.*, 2009, Goemans *et al.*, 2011, Cirak *et al.*, 2011). Intragroup variability was lowest with the model MS group which is the only group to be comprised entirely of patients harbouring the same deletion (exons 45 – 55). However moderate variability was observed in dystrophin and DAPC levels between patients of the same deletion.

The two different deletions within the model 51 group have comparable dystrophin and DAPC expression levels as well as a mild clinical phenotype. There is a greater variability in the clinical phenotypes between the patients of the model 53 group; our results also indicate a possible benefit for the retention of the nNOS-binding domain in internally deleted dystrophin.
We observed variability in dystrophin protein levels between patients with the same deletion; this could stem from the differences of individual intronic breakpoints in patients which may affect alternative splicing and/or translation efficiency. Other explanations for intra and intergroup variability could be the differential stability of the internally deleted dystrophins (Henderson et al., 2011, Krieger et al., 2010) and/or the endogenous splicing of other exons. For example exon 44 is known to spontaneously skip in patients with deletions of the surrounding exons (van Vliet et al., 2008). Although we have not measured this in our population, its occurrence could affect the levels of the dystrophin protein observed. Other genetic modifiers of dystrophin translation efficiency could also be involved in these discrepancies; for example a mutation in the promoter region of the gene encoding the extracellular matrix protein, osteopontin, identified osteopontin genotype as a genetic modifier of DMD severity (Pegoraro et al., 2011). Additionally the microRNA, miR-31, was recently shown to repress dystrophin expression through binding to the 3-untranslated region of dystrophin RNA (Cacchiarelli et al., 2011).

The model 51 group contained three out of the four asymptomatic patients whilst the model 53 group consisted of severe, mild and asymptomatic patients. Thus there is no clear-cut genotype-phenotype correlation in this latter group of BMD patients. We do however demonstrate for the first time that in BMD, higher expression levels of dystrophin, BDG and nNOS are significantly associated with a milder skeletal muscle phenotype.

Our results suggest that the milder phenotypes in our cohort of BMD patients are associated with higher expression levels of dystrophin and those DAPC members that bind directly to dystrophin. We found that BDG and nNOS expression correlates with clinical severity whilst ASG expression does not. This could be due to the direct
binding of BDG and nNOS to dystrophin; whilst ASG binds dystrophin indirectly (Ervasti, 2007, Ozawa, 2010). Our findings are further supported by mouse model data which demonstrate that the skipping of exons that preserve the nNOS-binding domain results in a more favourable clinical outcome (Lai et al., 2009); this information should be considered when developing exon skipping strategies.

Whilst it is generally established that the 45 – 55 deletion is associated with a favourable prognosis, this deletion is also associated with XLDCM (Beroud et al., 2007, Ferreiro et al., 2009, Miyazaki et al., 2009, Nakamura et al., 2008). In our study, the four patients with the 45 - 55 deletion were classified as mild and none had developed DCM, including one patient who died at age 76.

In fact, a large percentage of BMD patients develop DCM (Bushby et al., 2003, Melacini et al., 1993, Melacini et al., 1996) and mutations that disrupt the phasing of the helical spectrin repeats encoded by exons 45 - 49 are thought to lead to an earlier-onset of DCM as a result of altered dystrophin structure (Kaspar et al., 2009). The majority of the internally deleted dystrophin proteins studied within our cohort additionally remove the proline-rich hinge 3 (H3) region encoded by exons 50 and 51; the H3 region is thought to be helical but does not necessarily form a stable tertiary structure (Bhasin et al., 2005). One study designed nano-constructs of dystrophin based on in-frame BMD and therapeutic deletions including exons 45 – 51 (Krieger et al., 2010); our cohort included three patients with this deletion in the model 51 group. The secondary structure prediction for the abnormal “linker” created by the joining of exons 44 to 52 revealed that this region folds into a stable repeat domain that maintains its helicity (Krieger et al., 2010). These findings and our results provide further encouragement and guidance for exon skipping within the rod domain. However, a recent study has demonstrated that the removal of hinge 2 through to
spectrin-like repeat 19 causes a significant loss in protein stability (Henderson et al., 2011); thus caution is needed when removing large segments of the rod domain such as with multi-exon skipping models. Multi-exon skipping is also technically very challenging; only very minimal skipping has been detected at low frequency in preliminary cell culture work with a cocktail of antisense oligonucleotides for skipping of exons 45–55 (van Vliet et al., 2008).

It has been hypothesised that BMD patients with rod domain deletions suffer muscle cramps following exercise due to the mislocalisation of nNOS from the sarcolemma with subsequent disruption of blood flow during exercise (Kobayashi et al., 2008, Sander et al., 2000, Thomas et al., 1998). Our quantitative analysis of 17 BMD patients does not completely support this hypothesis. Firstly, several patients with deleted nNOS-binding domains and/or low sarcomemal expression levels do not suffer cramps and perhaps more convincingly, BMD patients 5, 7 and 8 report cramps even though their deletions do not span the nNOS-binding domain. In fact, patients 5 and 8 have near normal nNOS expression levels. The aetiology of cramps in BMD is complex and not necessarily or exclusively related to blood flow but to physical exercise. Thus mild individuals with preserved strength may have frequent cramps because they endure more physical activity, on the other hand growth and development may also play a role. A limitation of our conclusion is the relatively young age of a number of patients and we cannot rule out the possibility that some patients might develop cramps later on in life. Moreover, the recruitment of nNOS to the sarcolemma might also be affected by structural abnormalities located outside the nNOS-binding domain. Indeed, a complex correlation between sarcomemal nNOS localisation and muscle activity, oxidative stress and calcium signalling has been recently demonstrated (Finanger Hedderick et al., 2011, Pietri-Rouzel et al., 2010),
suggesting the presence of multiple independent regulators of nNOS localisation in muscle.

In summary, we demonstrate that all patients with the genotypes we have studied in this report had BMD and not DMD, in keeping with the reading frame hypothesis. While this not surprising, concerns that a significant number of patients with these genotypes could have DMD or severe BMD have been raised, although the source of the information relied on old studies when MLPA was not available and the endpoints of the deletions were not systematically assessed (Yokota et al., 2009). In fact, a more recent analysis of the same Leiden database used by Yokota et al has demonstrated that all patients with in-frame 45-51 or 50-51 deletions have a mild BMD phenotype (Helderman-van den Enden et al., 2010). Our data is further supported by two other reports; firstly Beroud et al reported on the mild or asymptomatic phenotypes of 15 patients with a 45-55 in-frame deletion (Beroud et al., 2007) and secondly a study that focussed on DCM in BMD reported the mild BMD phenotype of 24 patients with in-frame deletions that fit into one of the three models used in our study (Kaspar et al., 2009).

We show that BMD patients who express the same internally deleted dystrophin as could be induced by exon skipping therapies are mostly associated with mild phenotypes and express dystrophin at a high enough level (at least 40% of control) to provide a functional benefit to DMD patients. We report that the asymptomatic BMD phenotype is associated with significantly higher dystrophin, BDG and nNOS expression levels than the mild phenotype and highlight exon 51 as an optimal target exon for removal. This information is encouraging, as the dystrophin levels obtained in at least some of the patients recruited into the recently completed systemic clinical trials had levels of 15% (Goemans et al., 2011) and 18% (Cirak et al., 2011) of
normal levels; the latter representing approximately 45% efficacy during a short 12 week study, suggesting that a significant clinical benefit from these dystrophins is a realistic possibility.
Acknowledgements

The authors wish to thank the participating subjects and their families, the charities Muscular Dystrophy Campaign, Action Duchenne and the Duchenne Family Support Group for participating in the UK MDEX consortium (www.mdex.org.uk) which performed this study. We also gratefully acknowledge the support of the Duchenne Parent Project, Italy (DMD/BMD National Registry) and the TREAT-NMD neuromuscular network. We thank Ms Christa de Winter, Dr Valeria Ricotti and Mr. Darren Chambers for their assistance in immunohistochemical analysis and the professors Gert Jan B Van Ommen and Johan T. Den Dunnen for constructive discussions. We also wish to thank the MRC Neuromuscular Centre Biobank and Prof. Glenn Morris, Oswestry, U.K. and the MDA Monoclonal Antibody Resource for supplying the MANDSY106 antibody.

Funding

The work was supported by a Medical Research Council (MRC) grant to FM and by the MRC Centre for Neuromuscular diseases at UCL and Newcastle including the MRC Neuromuscular Centre Biobank. JM is supported by a Wellcome Trust University Award. FM is supported by the Great Ormond Street Hospital Children’s Charity.
Figure legends

Figure 1. Comparative immunohistochemical analysis of dystrophin expression in 17 Becker muscular dystrophy (BMD) patients with in-frame deletions. Control, DMD and BMD transverse muscle sections were immunolabelled for β-spectrin and with MANDYS106 (exon 43) and Dys2 (C-terminal, last 17 amino acids) antibodies against dystrophin. Expression was quantified relative to control muscle in 40 muscle fibres and normalised to β-spectrin expression. Values represent means ± SEM except for the far right graphs where values represent the mean expression level for each group ± SD of the difference between sample means. BMD patients are grouped according to corresponding exon skipping models for DMD: exon 51 skipping (model 51, red bars), multi-exon skipping (model MS, blue bars) and exon 53 skipping (model 53, yellow bars).

Figure 2. Representative immunohistochemistry images of dystrophin and dystrophin-associated proteins in Becker muscular dystrophy (BMD) patients with in-frame deletions. Unfixed, frozen transverse muscle sections (7 µm) from control, DMD and BMD (patients 8, 11 and 16 are shown) patients were immunolabelled with MANDYS106 (M106, exon 43) and Dys2 (last 17 amino acids of the C-terminus) antibodies against dystrophin and with antibodies against α-sarcoglycan (ASG), β-dystroglycan (BDG), neuronal nitric oxide synthase (nNOS) and β-spectrin (SP). Scale bar = 20 µm.

Figure 3. Western blot analysis of dystrophin expression in Becker muscular dystrophy (BMD) patients with in-frame deletions. A. Western blot of total
protein extracts from skeletal muscle biopsy of control and BMD patients. Blots were probed with the C-terminal Dys2 antibody for dystrophin and a sarcomeric α-actinin antibody as a loading control. **B.** Western blot semi-quantification using Image J software. Data is normalised to α-actinin and both individual and grouped patient data is presented as percent of control. Immunohistochemistry analysis of the same patients is presented alongside for comparison. Values represent means; for the graph of individual patients the error bars signify the SEM and for the grouped sample graph the error bars represent the SD of the difference between sample means. **WB =** western blot, **IHC =** immunohistochemistry.

**Figure 4.** Comparative immunohistochemical analysis of dystrophin-associated protein expression in 17 Becker muscular dystrophy (BMD) patients with in-frame deletions. Control, DMD and BMD transverse muscle sections were immunolabelled with antibodies against α-sarcoglycan (ASG), β-dystroglycan (BDG), neuronal nitric oxide synthase (nNOS) and β-spectrin. Expression was quantified relative to control muscle in 40 muscle fibres and normalised to β-spectrin expression. Values represent means ± SEM except for the far right graphs where values represent the mean expression level for each group ± SD of the difference between sample means. BMD patients are grouped according to corresponding exon skipping models for DMD: exon 51 skipping (model 51, red bars), multi-exon skipping (model MS, blue bars) and exon 53 skipping (model 53, yellow bars).

**Figure 5.** Correlation of dystrophin and dystrophin-associated protein expression with clinical severity. Clinical severity correlated to dystrophin expression with both MANDYS106 (red) and Dys2 (black) antibodies (A), ASG (B),
nNOS (C) and BDG (D) expression. Lines represent mean expression of each group. $p$ values are shown for statistically significant differences and were determined using a two-tailed Mann-Whitney test ($* p<0.05$ ** $p<0.01$ *** $p<0.001$).
<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Deletion</th>
<th>Centre</th>
<th>Biopsy</th>
<th>Age at study (yrs)</th>
<th>Age at onset of symptoms (yrs)</th>
<th>Symptoms at onset</th>
<th>Cramps</th>
<th>Myoglobinuria</th>
<th>DCM</th>
<th>Family history</th>
<th>Ambulant</th>
<th>Alive</th>
<th>Additional comments</th>
<th>Severity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51</td>
<td>45-51</td>
<td>L</td>
<td>Q</td>
<td>12</td>
<td>12</td>
<td>Toe walking, falls</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>y</td>
<td>y</td>
<td>y</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>51</td>
<td>45-51</td>
<td>LE</td>
<td>TA</td>
<td>48</td>
<td>4</td>
<td>Calf hypertrophy and cramps</td>
<td>y</td>
<td>n</td>
<td>n</td>
<td>y (grandfather)</td>
<td>y</td>
<td>y</td>
<td>Calf hypertrophy and childhood cramps, intolerance to exercise</td>
</tr>
<tr>
<td>3</td>
<td>51</td>
<td>45-51</td>
<td>R</td>
<td>Q</td>
<td>23</td>
<td>n.a</td>
<td>Incidental discovery of high CK</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>y (uncle)</td>
<td>y</td>
<td>y</td>
<td>Diagnosis made due to high CK</td>
</tr>
<tr>
<td>4</td>
<td>51</td>
<td>48-51</td>
<td>L</td>
<td>Q</td>
<td>10</td>
<td>2</td>
<td>Delayed motor milestones</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>y</td>
<td>y</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>51</td>
<td>48-51</td>
<td>N</td>
<td>Q</td>
<td>12</td>
<td>2</td>
<td>Muscle cramps and myalgia</td>
<td>y</td>
<td>n</td>
<td>n</td>
<td>y</td>
<td>y</td>
<td>Learning difficulties</td>
<td>Mild</td>
</tr>
<tr>
<td>6</td>
<td>51</td>
<td>48-51</td>
<td>F</td>
<td>TA</td>
<td>25</td>
<td>n.a</td>
<td>Incidental discovery of high CK</td>
<td>n</td>
<td>n</td>
<td>Mild</td>
<td>n</td>
<td>y</td>
<td>Epilepsy, during hospitalisation high CK detected</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>7</td>
<td>51</td>
<td>48-51</td>
<td>R</td>
<td>Q</td>
<td>16</td>
<td>8</td>
<td>Fatigue, no weakness</td>
<td>y</td>
<td>n</td>
<td>n</td>
<td>y (brother, uncle)</td>
<td>y</td>
<td>y</td>
<td>Mild TA contractures; calf and quadriceps hypertrophy</td>
</tr>
<tr>
<td>8</td>
<td>51</td>
<td>48-51</td>
<td>R</td>
<td>D</td>
<td>34</td>
<td>n.a</td>
<td>Incidental discovery of high CK</td>
<td>y</td>
<td>n</td>
<td>n</td>
<td>y (cousin)</td>
<td>y</td>
<td>y</td>
<td>Diagnosis made due to family history and high CK</td>
</tr>
<tr>
<td>9</td>
<td>MS</td>
<td>45-55</td>
<td>R</td>
<td>Q</td>
<td>34</td>
<td>4</td>
<td>Fatigue, cramps after exercise</td>
<td>y</td>
<td>y (frequently)</td>
<td>n</td>
<td>y (grandfather, patient 10)</td>
<td>y</td>
<td>y</td>
<td>Migraine; calf hypertrophy</td>
</tr>
<tr>
<td>10</td>
<td>MS</td>
<td>45-55</td>
<td>R</td>
<td>D</td>
<td>76</td>
<td>55</td>
<td>Walking difficulties</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>y (grandson, patient 9)</td>
<td>n (age 73, femur fracture)</td>
<td>n (2003age 76)</td>
<td>Stroke (1996); hypertension; diabetes; peripheral arteriopathy</td>
</tr>
<tr>
<td>11</td>
<td>MS</td>
<td>45-55</td>
<td>R</td>
<td>Q</td>
<td>23</td>
<td>4</td>
<td>Fatigue, mild difficulty in running</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>N</td>
<td>y</td>
<td>Neck flexors weakness</td>
<td>Mild</td>
</tr>
<tr>
<td>12</td>
<td>MS</td>
<td>45-55</td>
<td>R</td>
<td>Q</td>
<td>29</td>
<td>9</td>
<td>Myalgia, myoglobinuria</td>
<td>y</td>
<td>y</td>
<td>n</td>
<td>y (two uncles)</td>
<td>y</td>
<td>y</td>
<td>Calf and quadriceps hypertrophy</td>
</tr>
<tr>
<td>13</td>
<td>55</td>
<td>42-53</td>
<td>L</td>
<td>Q</td>
<td>14</td>
<td>4</td>
<td>Learning difficulties, ADHD</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>y</td>
<td>Normal muscle function, only high CK</td>
<td>Mild</td>
</tr>
<tr>
<td>14</td>
<td>55</td>
<td>45-53</td>
<td>L</td>
<td>Q</td>
<td>25</td>
<td>Un</td>
<td>Unable to run</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>y</td>
<td>Significant proximal weakness, Gower’s time 3 sec, inability to run, positive trendeleburg sign</td>
<td>Severe</td>
</tr>
<tr>
<td>15</td>
<td>55</td>
<td>45-53</td>
<td>L</td>
<td>Q</td>
<td>21</td>
<td>10</td>
<td>Myalgia</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>y</td>
<td>y</td>
<td>Calf hypertrophy</td>
<td>Mild</td>
</tr>
<tr>
<td>16</td>
<td>55</td>
<td>45-53</td>
<td>L</td>
<td>Q</td>
<td>14</td>
<td>n.a</td>
<td>Incidental finding of high CK</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>y</td>
<td>y</td>
<td>Mild TA contractures</td>
<td>Mild</td>
</tr>
<tr>
<td>17</td>
<td>55</td>
<td>52-53</td>
<td>F</td>
<td>TA</td>
<td>13</td>
<td>n.a</td>
<td>Incidental finding of high CK</td>
<td>n</td>
<td>n</td>
<td>n</td>
<td>y</td>
<td>y</td>
<td>Asymptomatic, only high CK</td>
<td>Asymptomatic</td>
</tr>
</tbody>
</table>
Table 1: Summary of clinical features. Centres: L = London, LE = Leiden, R = Rome, N = Newcastle, F = Ferrara. Muscle biopsies: Q = quadriceps, D = deltoid, TA = tibialis anterior. n = no, y = yes, un = unavailable, n.a = not applicable, DCM = dilated cardiomyopathy, CK = phosphocreatine kinase. Severity classification was based on the skeletal muscle phenotype.
References


Le Rumeur E, Winder SJ, Hubert JF. Dystrophin: more than just the sum of its parts. Biochim Biophys Acta. 2010 Sep;1804(9):1713-22.


spectrometry mapping of most exons and cooperative domain designs based on single molecule mechanics. Cytoskeleton (Hoboken). 2010 Dec;67(12):796-807.


