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Immunohistological intensity measurements as a tool to assess sarcolemma- associated protein expression

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Abstract

Aims: The quantification of protein levels in muscle biopsies is of particular relevance in the diagnostic process of neuromuscular diseases, but is difficult to assess in cases of partial protein deficiency, particularly when information on protein localization is required. The combination of immunohistochemistry and western blotting is often used in these cases, but is not always possible if sample is scarce. We therefore sought to develop a method to quantify relative levels of sarcolemma-associated proteins using digitally captured images of immunolabelled sections of skeletal muscle.

Methods: To validate our relative quantification method, we labelled dystrophin and other sarcolemmal proteins in transverse sections of muscle biopsies taken from Duchenne Muscular Dystrophy (DMD) and Becker Muscular Dystrophy (BMD) patients, a manifesting carrier of DMD and normal controls.

Results: Using this method to quantify relative sarcolemmal protein abundance, we were able to accurately distinguish between the different patients on the basis of the relative amount of dystrophin present.

Conclusions: This comparative method adds value to techniques that are already part of the diagnostic process and can be used with minimal variation of the standardised protocols, without using extra amounts of valuable biopsy samples. Comparative quantification of sarcolemmal proteins on immunostained muscle sections will be of use to establish both the abundance and localization of the protein. Moreover, it can be applied to assess the efficacy of experimental therapies where only partial restoration or upregulation of the protein, may occur.

Key words: Protein quantification; Immunohistochemistry; Genetic therapies; BMD: Becker muscular dystrophy ; DMD: Duchenne muscular dystrophy

Introduction

The study of proteins expressed either at the muscle fibre plasmalemma, or in the basal lamina extracellular matrix, is the basis for the diagnosis of a number of muscular dystrophies. These include DMD, characterized by the absence of the sarcolemmal- associated cytoskeletal protein dystrophin, congenital muscular dystrophy MDC1A, due to the deficiency of the extracellular matrix protein laminin α2, and Ullrich CMD, due to reduced collagen VI [1]. However, in some of these conditions the protein deficiency is subtle, and can be difficult to evaluate. Moreover, in some muscular dystrophies the patterns of secondary protein changes can aid in the diagnostic process [1]. Examples of these are cases of utrophin upregulation in dystrophinopathies [2]; dystrophin reduction in some sarcoglycanopathies [3, 4]; absent

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nitric oxide synthase in DMD and some BMD patients [5, 6]; reduced laminin α2 in alpha dystroglycanopathies [7, 8] or increases in laminin α5 in MDC1A and dystroglycanopathies [9]. The quantitative study of the expression of these proteins, and their localization, is also vital for the correct assessment of experimental strategies designed to restore the missing protein in adequate amount, in the correct localization and interacting appropriately with other proteins in order to restore muscle function.

Immunohistochemical techniques are frequently used to study the abundance and localization of proteins associated with these diseases [10]. Western blot analysis is also of use in the diagnosis of patients affected by muscular dystrophies, offering valuable semi-quantitative data [11]. However, this technique requires greater amounts of sample and volume of antibodies and it only offers true quantitative information when studying samples far from the low and high detection limits [11, 12]. Furthermore, in diseases like Ullrich congenital muscular dystrophy (UCMD), where a reduction in collagen VI in the basal lamina rather than the interstitial connective tissue is a feature, reliable quantitative information of basal lamina protein levels is crucial [13].

In order to combine information on protein localisation and abundance, we sought to develop a reproducible method to be able to quantitatively measure protein abundance in immunohistochemical labelled skeletal muscle. As many of the disease-relevant proteins are currently analysed on muscle sections using a standard diagnostic antibody panel [1], this technique to quantify relative sarcolemmal protein abundance could be undertaken with minimal variation of the standardised protocols and without the use of extra amounts of valuable sample.

To validate this method, we compared the amount of dystrophin in muscle samples from a number of patients with different levels of dystrophin expression:

a) DMD patients, in whom mutations in the *DMD* gene which disrupt the reading frame and prevent production of functional dystrophin [12, 14].
b) Becker Muscular Dystrophy (BMD) patients, with allelic mutations in the *DMD* gene which maintain the reading frame giving rise to shorter but semi-functional dystrophins and a milder phenotype [12, 15, 16]. These internally deleted dystrophins, likely to be less stable compared to wild type protein, result in reduced, but variable, protein levels in these patients.
c) Manifesting female carriers of DMD, with a mosaic expression of dystrophin negative and positive fibres, due to different X-inactivation in different myonuclei [17, 18].
d) Control individuals, with no known neuromuscular disorder and had normal dystrophin expression.

Materials and Methods

Histology and Immunocytochemistry.

Skeletal muscle biopsies were obtained with informed consent from patients with DMD (n=8), BMD (n=1), normal controls (n=5) and a manifesting carrier of DMD (n=1) (Table 1). All boys with DMD followed a typical clinical course; the BMD patient (in frame deletion 45-47) was a mild case: currently 8 years old, is able to walk for long distances, run and hop. The clinical severity of the manifesting carrier is moderate with clear symptoms mostly related to pain and fatigability, her main limitation being muscle cramps when walking.

Samples from the quadriceps muscle (minimum sample size 4x3x3mm) were obtained using a needle technique at the Dubowitz Neuromuscular Centre in Hammersmith Hospital, London, recently relocated to the Institute of Child Health & Great Ormond Street Hospital for Children, London. Samples from the extensor digitorum brevis (EDB) and paraspinal muscles were obtained at the Royal National Orthopaedic Hospital in Stanmore, UK, during foot and scoliosis surgery. Control paraspinal samples

were obtained from patients with adolescent idiopathic scoliosis during their scoliosis surgery. Ethical approval for this project was granted by the Multi-centre Research Ethics Committee (*MREC*) in UK. Muscle biopsies were rapidly frozen in isopentane cooled in liquid nitrogen according to standard techniques. Unfixed frozen transverse sections (7 µm) were incubated with primary antibodies for 1 hr at room temperature. Following three washes in PBS, sections were incubated with biotinylated secondary anti-mouse or anti-rabbit antibodies (Amersham UK, 1:200) for 1 hr at room temperature. Samples were then incubated with streptavidin conjugated to Alexa 594 (Invitrogen UK, 1:1000 for 15 min at room temperature and washed in PBS before mounting in Histomount (National Diagnostics). The antibodies used were: Dys 2 (1:20) and P7 (1:1000) (against dystrophin exons 77-79 and 57-60 respectively) (15, 16), β-dystroglycan (BDG)(1:20), α-sarcoglycan (ASG) (1:50), spectrin (SP)(1:20), and utrophin (UTR)(1:5). All primary antibodies except P7 were monoclonal and obtained from Vision Biosystems, UK. P7 was a rabbit polyclonal antibody produced against the same sequence as Sherrat et al. [19].

Intensity measurements

Sections from the biopsies were immunolabelled and evaluated using a Leica DMR microscope interfaced to Metamorph (Molecular Devices, US). Control muscle sections, expressing normal levels of dystrophin, were immunostained simultaneously and used to set the exposure values for the DMD samples for each of the antibodies used except for utrophin, for which the exposure settings were those of one of the DMD samples. Four images of different sectors of the section selected at random while out of focus were focused, captured and analysed from each the sample. From each image, ten different regions were randomly selected. However, if the region was in the centre of the fibre, on an area of fibrosis, on a neuromuscular junction or if more than one measurement per fibre was selected, the region was moved slightly to the nearest fibre membrane. The measured regions included both a portion of the cytoplasm and the sarcolemma (Figure 1A).

The principles of this technique are the following: when excited, fluorescent labelled antibodies bound to the proteins release photons that are captured by the Charge Coupled Device (CCD), converted into electrons. The number of electrons, which is directly proportional to the intensity of the fluorescence, are then mapped on to an image in MetaMorph and presented as an intensity value (Figure 1B and C). The dynamic range of the camera (a 12 bit Photometrics CoolSnapHQ2) was 0-4095 intensity units and our measurements were taken so that pixel saturation was avoided (all our intensity measurements were well below the saturation limit). Intensity measurements of these regions were logged into a spreadsheet for data analysis. For each antibody used, 40 different measurements from each sample were taken.

Patient	***Diagnosis***	***Biopsy***
1	Control (normal dystrophin)	Quadriceps
2	DMD Deleted exons 50-53	Quadriceps
3	DMD Deleted exons 46-52	Quadriceps
4	DMD Deleted exons 3-13	Quadriceps
5	DMD Deleted exon 44	Quadriceps
6 Quad	DMD Deleted exons 45-52	Quadriceps
6 EDB	DMD Deleted exons 45-52	EDB
7	Control (idiopathic scoliosis)	Paraspinal
8	DMD Deleted exons 46-49	Quadriceps
9	BMD Deleted exons 45-47	Quadriceps
10	Manifesting Carrier	Quadriceps
11	Control (idiopathic scoliosis)	Paraspinal
12	Control (idiopathic scoliosis)	Serratus
13	Control (idiopathic scoliosis)	Paraspinal
14	Control (idiopathic scoliosis)	Intercostal
15 Quad	DMD Stop mutation in exon 70	Quadriceps
15 EDB (R)	DMD Stop mutation in exon 70	Right EDB
15 EDB (L)	DMD Stop mutation in exon 70	Left EDB

Table 1 Diagnosis, and muscle type of the samples used in this study.

Data analysis

Each region where intensity values were measured contained a portion of the cytoplasm and of the sarcolemma, reflecting the location of the proteins of interest. For each region, the minimum intensity value recorded (representative of the cytoplasm or background intensity) was subtracted from the maximum intensity value (which corresponded to the sarcolemma) to correct each measurement for background intensity. To correct for variation of sarcolemmal integrity between samples, we performed the same measurements on serial sections stained with a β-spectrin antibody. The spectrin intensity values obtained for the control samples were set as the standard to calculate normalization factors.

For each of the antibodies, the minimum intensity value was subtracted from the maximum, then these values (one per each of the 40 fibres analysed) were normalized with the β-spectrin measurements and plotted on a graph. Data are presented in scatter plots and summarized as a ratio of the control.

Statistical analysis of the data was performed using one-way analysis of the variance.

Results

Intensity measurements can distinguish dystrophin protein levels in DMD, BMD and manifesting carrier

We compared muscle sections taken from a normal control, a DMD patient, a BMD patient and a manifesting carrier, using two dystrophin antibodies (Dys2 and P7). We also studied in parallel the intensity of dystrophin-associated complex proteins (ASG, BDG) and UTR (Figure 2A). When a DMD sample was compared with a BMD and the standard control using Dys2 antibody, the DMD sample showed very low dystrophin intensity relative to the normal control (barely over the background level), while the BMD sample showed an intermediate intensity relative to the control (approximately 0.5 of the control values). When the same samples were studied with P7, an antibody to a different region of the dystrophin protein, the findings were comparable: DMD showed values close to 0.15 of the control, while the BMD sample was 0.6 (Figure 2A). In both cases, the differences between BMD and DMD samples were highly significant ($p<0.001$).

In both DMD and BMD muscles, a decrease in the associated proteins ASG and BDG was also detected (Figure 2A). While BDG intensity was similarly reduced both in DMD and BMD muscles (0.4 and 0.35 of the control) (Figure 2A), the BMD sample studied showed lower relative intensity of ASG than the DMD sample (0.15 and 0.4 of the control, respectively).

In cases of dystrophin deficiency, utrophin is up-regulated at the sarcolemma [2]. Our comparative intensity measurements confirmed this: sections of DMD muscles showed a marked increase in relative intensity compared to the control; the over-expression of utrophin was inversely correlated to the depletion of dystrophin (Figure 2). This over-expression was approximately five times the control in the DMD sample (the DMD sample was used as the reference for the capture settings), in which dystrophin was absent, and close to 3 times in the BMD sample. These differences were statistically significant ($p<0.001$).

The analysis of the manifesting carrier sample revealed mean dystrophin intensity measurements similar to those obtained from the BMD sample (Figure 2A). However, when studying the scatter plots for this sample, a very clear segregation of the fibres was evident. As sections of this sample showed a mosaic pattern of dystrophin expression, with some fibres staining strongly and others more weakly (Figure 1), the study was extended to select 100 measurements of strongly-labelling (bright) and 100 measurements of weakly-labelling (dim) fibres, instead of the usual random measurements. When these measurements were compared to control muscle, the weakly stained fibres showed values no significant difference to those in DMD samples, whereas the strongly staining fibres were not as bright as the control ($p<0.001$), but showed values of similar intensity as those observed in BMD samples (Figure 2B).

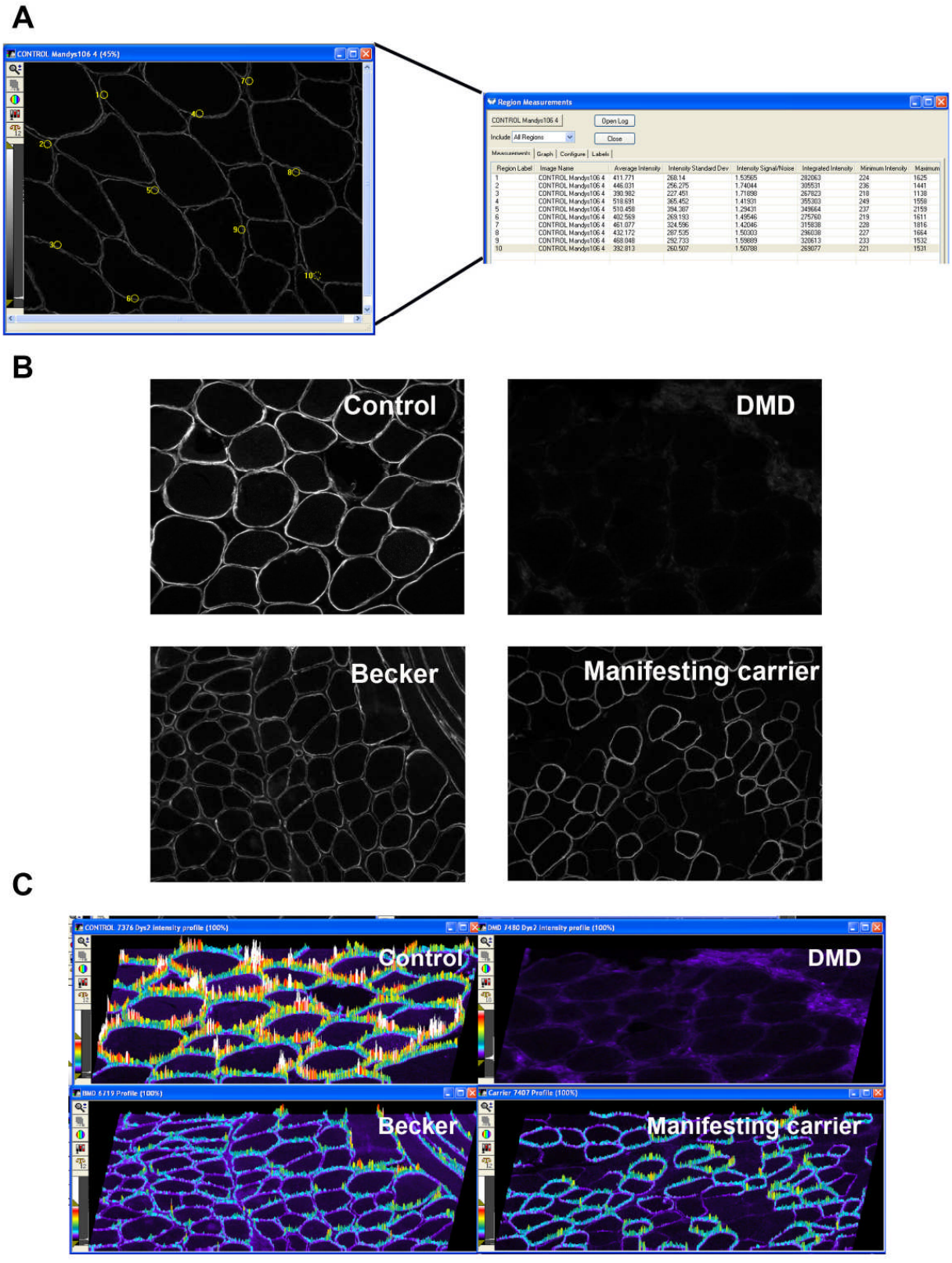


Figure 1 A) Example of the way regions were positioned in the image and how the intensity of those regions was recorded. Each of the ten regions (labelled in yellow in the left image) was measured to obtain the intensity measurements (right) used in this study. **B)** Transverse cryosections of quadriceps muscle biopsies, immunostained with Dys 2 antibody showing control (normal immunostaining), DMD, showing no dystrophin, BMD, showing decreased intensity, and a manifesting carrier, with characteristic mosaic dystrophin expression. **C)** The corresponding intensities profiles of the images in B, as they are detected by the Metamorph program.

Subtle differences in dystrophin intensity reported by pathologists are easily identifiable with this method.

In approximately 20% of DMD patients, traces of dystrophin- patches of below-normal dystrophin-positive areas visible at the sarcolemma of muscle fibres- are present [11]. The quantification of this low level of dystrophin expression by western blotting would require high amounts of sample [20]. To attempt to quantify traces of dystrophin with our method to quantify relative sarcolemmal protein abundance, sections from six DMD patients (Table 1) were stained with Dys2 antibody and results compared with the reports from the pathologist (CAS) who had previously reported trace expression of dystrophin in three of them. We found a complete concordance between our measurements and the pathologist's reports: those samples that showed higher relative intensity when analysed with our method were described in the report as showing traces, as opposed to complete absence, of dystrophin (Figure 3).While there were no significant differences between the samples containing traces (samples 3, 4 and 5), the differences between them and those without traces (samples 2, 6A and 6B) were highly significant ($p<0.001$).

Sample variability

To evaluate how much variability there is in the standard samples used as controls, a set of quadriceps muscle biopsies from 4 individuals without a neuromuscular disease were compared. While in three cases the analysis failed to show any significant difference between the samples analysed, muscle from one control showed significantly reduced dystrophin expression ($p<0.01$ or $p<0.05$ between control 11 and controls 12 and 14 in Dys2 analysis)(Figure 4A).

To determine if samples from different muscles of the same DMD patient contained similar levels of dystrophin, 3 samples from the same patient were compared (quadriceps sample taken at the time of diagnosis, right and left EDBs taken ten years later). All three samples showed very limited dystrophin intensity when analysed with both dystrophin antibodies (0.05 of control for Dys2 and 0.15 of control for P7), a similar decrease in the sarcolemmal associated proteins (BDG: 0.36 of control and ASG 0.65) and over-expression of utrophin to an equivalent level (approximately 6.5 times the intensity of the control) (Figure 4b). There was no statistically significant difference between any of these measurements.

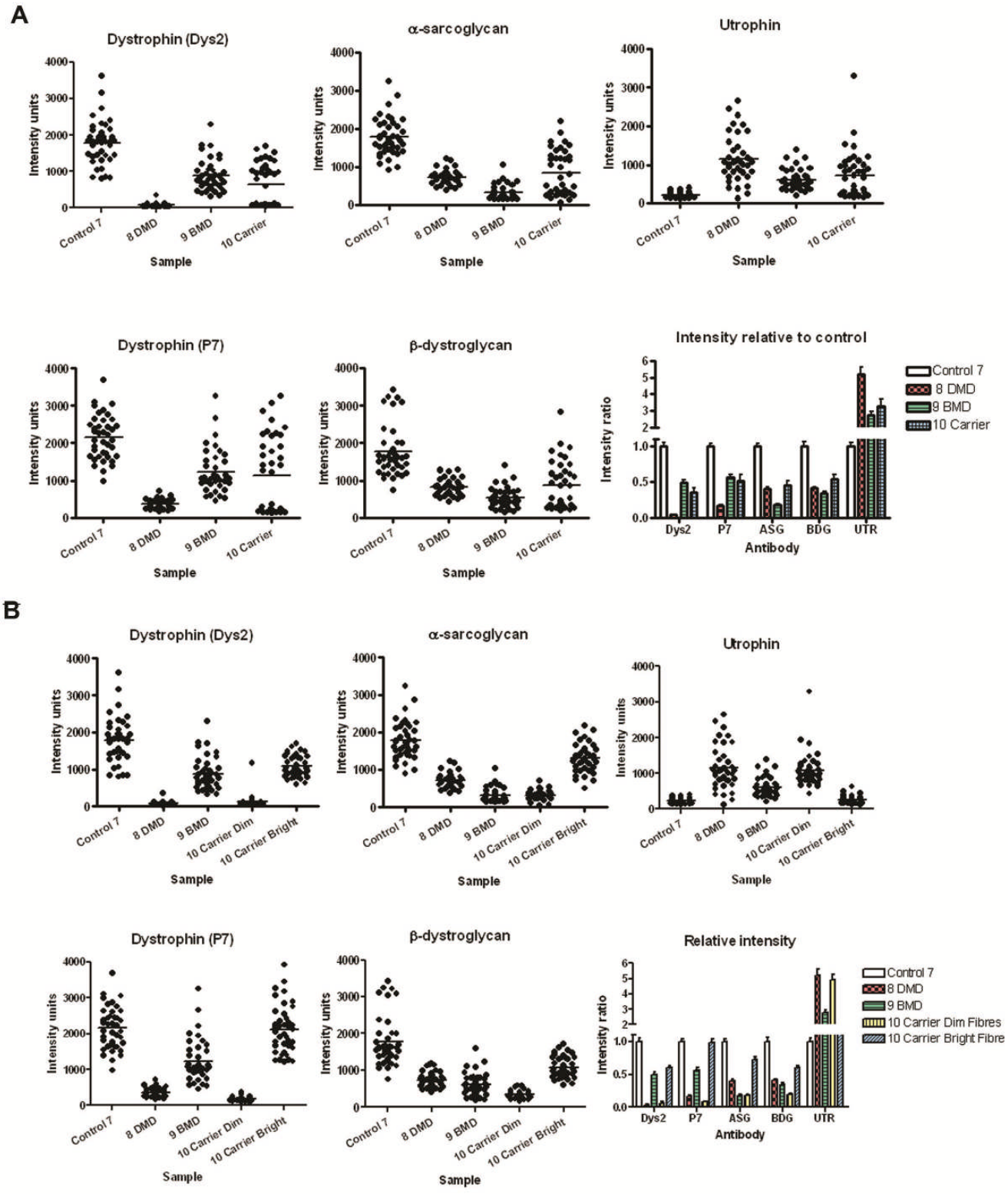
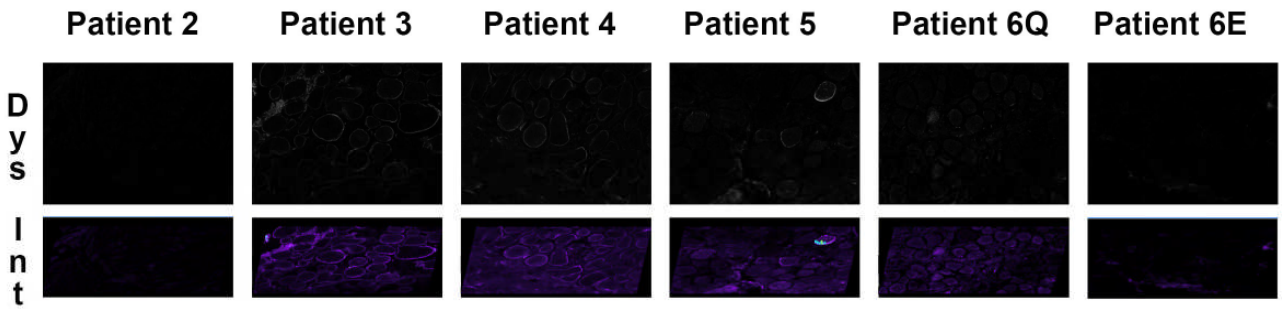


Figure 2

A) Comparative analysis of a control, DMD, BMD and a manifesting carrier muscle sample. **B)** The same data, once the manifesting carrier samples had been divided into strongly and weakly-labelling fibres.

A



B

Sample	Pathologist's report
2	No traces, no revertants
3	Traces, one revertant
4	Traces on most fibres
5	Traces, Five/six revertant fibres scattered through the sample
6 Quad	No traces, no revertants
6 EDB	No traces, one revertant

C

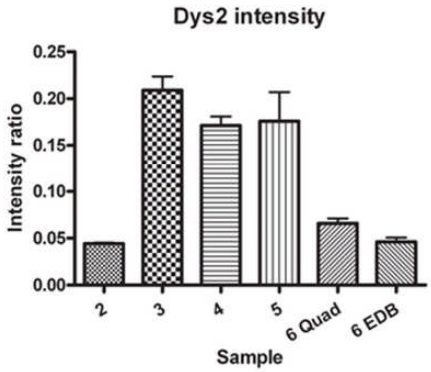


Figure 3 A) Images from sections stained with Dys2 antibody and their corresponding intensity profiles. **B)** Transcript from the pathologist reports corresponding to the samples in A and C. **C)** Relative intensity measurements obtained when this group of 6 DMD samples were compared against one control.

Discussion

A range of muscular dystrophies are routinely diagnosed by immunostaining muscle biopsies, sometimes in combination with western blotting analysis. Many of these disorders, such as DMD or BMD or UCMD, are characterised by reduced expression of sarcolemmal proteins, which is sometimes subtle [13]. Secondary protein changes also often occur [1], Quantification of protein expression from muscle biopsies is not trivial; while western blot analysis of serial dilutions of muscle lysate can provide semiquantitative analysis, it requires an amount of tissue that is not always available [20, 21].

In this study, we have compared the levels of dystrophin expression in muscle fibres of DMD, BMD, a manifesting carrier and patients with normal dystrophin expression.

We first used randomly-encountered regions of each image of immunostained muscle transverse sections to perform the analysis. This has the advantage of avoiding any bias from the operator, although can obviously miss discrete areas of relevance, e.g. clusters of revertant fibres in DMD [22, 23], or the mosaic dystrophin expression observed in DMD manifesting carriers [17, 24]. The mean relative dystrophin intensity of randomly-encountered fibres in a manifesting carrier was similar to a BMD patient (Figure 2A), but when the intensity of strongly and weakly labelled fibres was measured separately, the mosaic pattern of dystrophin expression characteristic of a manifesting carrier was readily quantifiable (Figure 2B). Similarly, when randomly analysing fibres from sections containing revertant fibres, either an increased average intensity, or higher standard errors of the mean was seen, implying that revertant fibre(s) had been included in the analysis (e.g. sample 5 in Figure 3).

As with any semi-quantitative technique, reliable internal controls and standards are vital. We chose β-spectrin as our internal control, to account for differences in the integrity of the fibres. We have previously

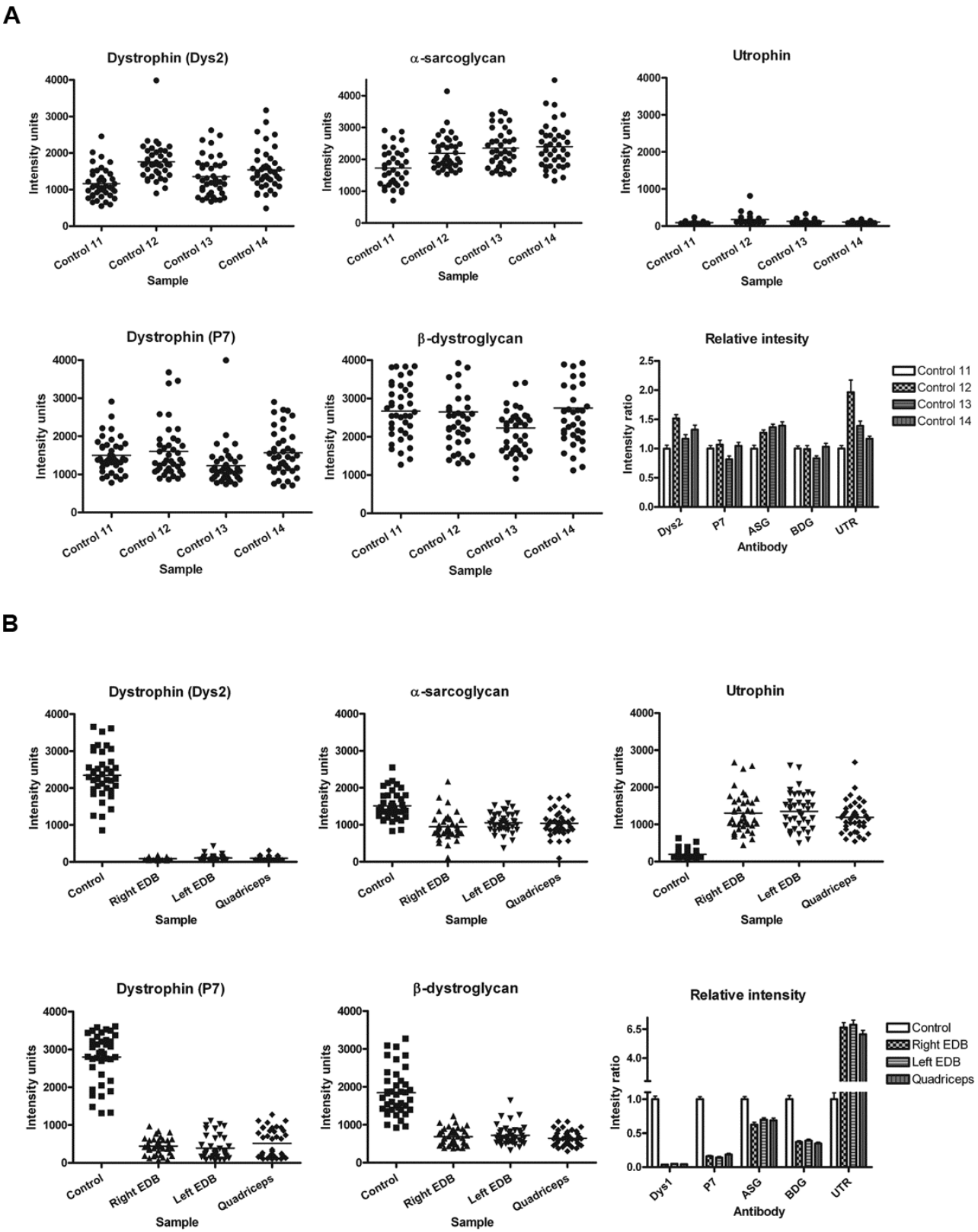


Figure 4 A) Comparative analysis of four different controls (scoliosis patients). **B)** Three samples from three different muscles of the same patient were analysed and compared with a control.

shown that spectrin is an ideal marker of sarcolemmal integrity as it is not a protein of the dystrophin complex [25] and is not affected by dystrophin deficiency, except on necrotic and regenerating fibres [26]. All measurements were normalised with their corresponding serial section labelled for β-spectrin. All measurements were expressed relative to the normal dystrophin in standard controls in each particular experiment and should not be considered absolute values, as we confirmed that there is a certain degree of variability even between controls (Figure 4). We believe that this technique will be an additional useful tool to the techniques currently in place in diagnosis of neuromuscular diseases in which the study of localization and amount of protein is paramount. We also propose this technique as an objective method to quantify protein expression when assessing efficacy of experimental therapies aimed at restoring protein expression such as in the recent trials of antisense oligonucleotides in DMD [27, 28].

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Author Disclosure Statement

No competing financial interests exist.

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