Integrated study of 100 patients with Xp21 linked muscular dystrophy using clinical, genetic, immunochemical, and histopathological data. Part 1. Trends across the clinical groups


Abstract

This multidisciplinary study was undertaken to record the variation in gene and protein expression in a large cohort of patients with well defined clinical phenotypes. The patients, whose ages ranged from 4 years to 66 years, spanned a wide range of disease severity. They represented the first 100 patients who had been examined in Newcastle, had undergone a muscle biopsy, and provided a blood sample for DNA analysis. The study had three aims: to observe any trends in the analyses across the clinical groups, to correlate gene and protein expression in individual patients, and to use the data collected to assess the relative usefulness of different techniques in the diagnosis and prognosis of patients with Duchenne and Becker dystrophy (DMD/BMD). In part 1, we describe the clinical assessment of the patients and the trends that were observed across the cohort. The patients were divided into seven groups. Group 1 had severe DMD (n=21), group 2 had milder DMD (n=20), group 3 were intermediate D/BMD patients (n=9), group 4 had severe BMD (n=5), and group 5 were more typical BMD patients (n=31). Some patients were too young to be classified (n=7) and a group of all the female patients were also classified separately (n=7). The number of DMD and BMD patients was about equal, in accord with disease prevalence in the north of England, but an unusually high proportion were sporadic cases. Dystrophin labelling (performed with up to three antibodies) on both blots and sections increased gradually across the clinical groups. All histopathological indices, except the proportion of fat in biopsy sections, showed clear trends across the groups.

(Med Genet 1993;30:728-36)

Duchenne and Becker muscular dystrophy (DMD, BMD) are both caused by defects in a gene on the X chromosome located at Xp21.1 The defects cause abnormal expression of the protein product, dystrophin, apparently in all patients.14 Most intragenic mutations are deletions, but duplications and point mutations have also been identified.5-7 The clinical spectrum of Xp21 linked muscular dystrophy varies from severe DMD, where the progressive loss of muscle function results in wheelchair dependency from about 7 years and death before 20 years, to mild BMD where a wheelchair may never be needed and a normal life span is frequently attained. In order to understand the pathogenesis of Duchenne and Becker dystrophy, the extent of variation in genotype and phenotype expression needs to be recorded. To this end we have undertaken a multidisciplinary investigation with the following three aims: to identify any patterns or trends in the analyses which would be shown by investigating a cohort of patients with a wide range of disease severity, to relate gene and protein defects in individual patients with well defined clinical phenotypes, and to identify which techniques are the most important for differential diagnosis and prognosis. The whole report has been divided into three sections, each addressing one of the aims defined above. In this first part we define the patient groups and report the pattern of results obtained across the clinical spectrum.

Materials and methods

PATIENT SELECTION

The 100 patients (93 male and seven female) included in this study were the first 100 patients with confirmed Xp21 linked muscular dystrophy who fulfilled the selection criteria of (1) being examined at the Regional Neurosciences Centre of Newcastle General Hospital, (2) having undergone a muscle biopsy (which was stored in a liquid nitrogen archive), and (3) having provided a blood sample for lymphocyte DNA analysis. A further requirement was that, as far as possible, the patients were not related. Nevertheless, the 100 patients represent 98 family lines: also included were a nephew and uncle from one severe BMD family (patients F58 and F59 in the appendix) and a BMD father and his obligate carrier daughter (patients F67...
and F94). The results from the genetic analysis were not known when the patient cohort was assembled but simple screening for dystrophin with a single antibody had been performed to confirm the initial clinical diagnosis.

CLINICAL ASSESSMENT
All patients in this study were examined by the same clinicians (DGM/KMDB). The following were recorded for each patient as appropriate: family history, age first walked, first symptoms, age first symptoms were observed, age at diagnosis, initial diagnosis, age at biopsy, age when ability to walk independently was lost, length of time in callipers (if used) before becoming wheelchair bound, length of time in wheelchair before death, age at death, IQ, age first words spoken, age first sentences constructed, achievements at school, further education, and employment undertaken. During the early years the times taken to run along a standard 11 m length of corridor, to climb six stairs, and to get up from the floor were recorded at every hospital visit. Measurements of forced vital capacity were also made at intervals following the loss of independent mobility. The disability of Becker patients was assessed according to the Cornelio scale.8,9 The strength of all the major muscle groups was tested and the results were related to age, a low score indicating little reduction in muscle function. Electrophysiological studies (EMG/ECG) had been performed on too few patients in this cohort for inclusion.

PATIENT CLASSIFICATION
The patients were basically classified according to the standard clinical criterion of the age at which independent mobility was lost and full length callipers or a wheelchair had to be used. Thus DMD patients lost the ability to walk independently by 12 years of age, intermediate D/BMD patients between 12 and 16 years, and BMD patients could walk independently until they were at least 16 years old. For the purposes of comparison in this investigation, however, the number of clinical groups was expanded. Among our entire population of DMD patients (not just those in this study) the average age of becoming wheelchair bound is just over 9 years. We therefore divided the DMD patients in this study into two groups: those in a wheelchair before 9 years (group 1, n = 21) and those who lost mobility between 9-1 and 12 years of age (group 2, n = 20). Patients with intermediate clinical symptoms (wheelchair bound 12-16 years) formed group 3 (n = 9). The BMD patients were also divided into two groups: those in a wheelchair between 16 and 40 years (group 4, n = 5) and those not in a wheelchair until past 40, if at all (group 5, n = 31). Patients who were still ambulatory were assigned to clinical groups according to muscle function tests: group 4 patients corresponding to the ‘severe Becker’ group and group 5 to the ‘typical Becker’ group differentiated on the basis of Cornelio muscle function analysis described in detail by Bushby and Gardner-Medwin in 1993.9 The females were considered together as a separate group (group F, n = 7). One (patient S95 in the appendix) was the single affected member of a pair of monozygous twins.10

The current age range in our cohort of patients is from 4 to 66 years. Some of these patients could still walk independently. For the purposes of this study, most of these patients could be assigned to a clinical group according to their rate of disease progression. Detailed records are kept of every visit to the Regional Neurosciences Centre made by patients with muscular dystrophy so that a very large data base has been generated over the last 30 years. Thus it was possible to plot, for example, the time taken at different ages to run along an 11 m corridor or to get up from the floor for a very large number of patients in whom the whole clinical course had subsequently been recorded. By comparing the early data on patients whose clinical course was known with data collected on young patients who had not yet lost independent mobility, most young patients could be assigned to one of the five clinical groups. Nevertheless, patients under 6 years of age were still considered too young for assignment (group TY, n = 7) and we readily acknowledge that it is not possible to predict a clinical course from early observations with complete accuracy. It must also be emphasised that patients with Xp21 linked muscular dystrophy present a continuous, though not evenly distributed, spectrum of disease severity and separation into five clinical groups was undertaken purely for comparative purposes in this study. The criteria used have no implications for classification for diagnostic purposes.

GENETIC ANALYSIS
Analysis of DNA isolated from lymphocytes and digested with the restriction enzyme HindIII was performed using dystrophin cDNA probes 1–2a, 2b–3, 4–5a, 5b–7, 8, 9–10, and 11–14 according to established techniques.6 In some instances PCR analysis was performed using multiplex primers.12 The genetic analysis for 50 patients was performed in Leiden, The Netherlands. Deletions and duplications could be detected but not point mutations or other very small genetic rearrangements. X chromosome inactivation studies were undertaken for the female patients using differential methylation and fluorescent in situ hybridisation techniques which have been described in detail previously.10,13

ANTIBODIES USED FOR PROTEIN ANALYSIS
Four monoclonal antibodies (MABS) were used during this study: three to dystrophin and one to β-spectrin. The rod and C-terminal dystrophin MABS detect epitopes on either side of the major deletion ‘hotspot’. Generation and use of the rod domain MAB, Dy4/6D3 (DYS1), has been described previously.14,15 The immunogen was the 30 kDa fusion protein reported by Hoffman et al16 and the epitope recognised is between amino acids 1181 and 1388 (exons 26 to 30 approximately). The C-terminal MAB, Dy8/6C5 (DYS2), was generated by immunisation with a synthetic
peptide consisting of the last 17 amino acids (3669–3685) of the human dystrophin sequence\(^{16}\) conjugated to keyhole limpet haemocyanin. The N-terminal MAb, Dy10/12B2 (DYS3), was generated by immunisation with a fusion protein containing cDNA C27,\(^9\) deletion analysis localised the epitope to the vicinity of exons 10 to 12, and subsequent epitope mapping with synthetic peptides (kit from Cambridge Research Biochemicals, UK) suggests that the antibody binds to sequences within amino acids 308 to 351 which span the junction of exons 9 and 10. This may be part of a hinge region joining the amino domain to the central rod domain\(^{18}\) and reactivity with a conformational epitope would explain why labelling with Dy10/12B2 is much weaker on blots but stronger on tissue sections than the other dystrophin MAb. None of the dystrophin MAb reacts with the chromosome 6 encoded dystrophin-like protein,\(^9\) also known as dystrophin related protein, DRP,\(^{20}\) or utrophin.\(^{21}\) The \(\beta\)-spectrin MAb, RBC2/3D5 (SPEC1), was generated by immunisation with human red blood cell membrane ghosts.\(^{22}\) Spectrin is localised at the periphery of muscle fibres like dystrophin and labelling of this protein is thus an excellent control for muscle membrane integrity in fibres which appear negative for dystrophin labelling. All four antibodies (DYS1, 2, 3, and SPEC1) can be obtained from Novocastra Laboratories, 22 Claremont Place, Newcastle upon Tyne NE2 4AA, UK.

**IMMUNOCYTOCHEMISTRY**

Details about the site, size, and freezing of blocks of tissue from muscle biopsies have been described previously.\(^{15}\) The immunocytochemical analysis was performed on 6\(\mu\)m unfixed frozen tissue sections. The indirect horseradish peroxidase technique was used with visualisation of the label achieved by exposure to \(\text{H}_2\text{O}_2/\text{diaminobenzidine}.\)\(^{15}\) All the biopsies were labelled with the rod MAb and in most cases serial sections were labelled with the C-terminus MAb. The amino-terminal MAb was used in a few cases when, for example, a patient had a deletion in the rod domain that removed the Dy4/6D3 binding site. The immunocytochemical labelling patterns were divided into eight categories according to the most prominent features observed: ICC 1 – no labelling on any fibres; ICC 2 – faint labelling on occasional fibres, rest negative; ICC 3 – clear labelling on a few fibres (generally <1\%), rest negative; ICC 4 – labelling of severely decreased intensity on up to 50% of fibres, a few may be more intensely labelled, rest negative; ICC 5 – labelling of severely decreased intensity on nearly all fibres; ICC 6 – labelling shows variation between and/or within fibres; ICC 7 – labelling is uniform but decreased in intensity; ICC 8 – labelling at near normal intensity (indistinguishable from controls). Figures illustrating labelling patterns like these may be found in previous papers.\(^{15,23}\)

**WESTERN BLOTTING**

Sample preparation, polyacrylamide gel electrophoresis (4 to 7\% gradient resolving gel, 3\% stacking gel), and Western blotting were performed as described previously\(^{14}\) as were the estimates of dystrophin size\(^{24}\) and abundance.\(^{15,22}\) Dystrophin abundance was estimated by scanning densitometry of the myosin staining on post-blotted gels and the uppermost dystrophin band labelled in each blot lane. Thus, dystrophin abundance (expressed as a percentage of normal) could be adjusted for the amount of muscle protein (as opposed to fat and fibrous connective tissue) in the weight of sample homogenised. The principle of the densitometric analysis has been described in detail previously.\(^{27}\) All biopsies were labelled with the rod MAb, most with the C-terminal MAb, and a few with the N-terminal MAb as in the immunocytochemical analysis.

**HISTOPATHOLOGY**

For histopathological examination frozen tissue sections 10\(\mu\)m thick were cut, post-fixed in formal calcium, and stained with haematoxylin and eosin. The percentage area of each biopsy occupied by fat and fibrous connective tissue was measured using a conventional stereological (point counting) technique.\(^{28}\) Photomicrographs of tissue sections printed at a constant \(\times 150\) magnification were used. Four areas per biopsy were analysed comprising a total of 5\(\,\text{mm}^2\) except when the size of the biopsy did not permit this. Control data derived from a series of patients with no histological evidence of neuromuscular disease showed that the normal upper limits of fibrous connective tissue and of fat in skeletal muscle are about 12\% and 5\% respectively. The incidence of features which indicate acute histopathological change (hyaline fibres, necrotic fibres, regenerated fibres), and those which indicate chronic change (ring fibres and fibre splitting) were estimated semi-quantitatively. Four categories of severity were defined for each histological feature: 0 = nil, 1 = <2\%, 2 = 2–5\%, and 3 = >5\%. The incidence of groups of fibres showing degeneration and regeneration was also assessed. To circumvent the problem of age related changes in the absolute size of muscle fibres, the range of fibre diameters was expressed as n-fold variation (for example, 5-fold, 10-fold). The normal upper limit of fibre size variation is 3-fold. The histopathological and immunocytochemical analyses were performed on all the biopsies by the same person (MAJ).

**STATISTICAL ANALYSIS**

Each person responsible for an aspect of this study completed collection of their data without knowledge of all the results from other participants. The data were entered into a database programme and analysed only at the completion of the study. The values for dystrophin size and abundance given in the appendix are the final numbers that were arrived at after the calculations described above and have not been simplified. Tests used were the Student \(t\) test, paired \(t\) test, \(\chi^2\) test, one way analysis of variance (ANOVA) followed by multiple comparison tests to identify individual differences, and regression analysis. A \(p\) value of greater than 0.05 was considered to be 'not significant'.
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Results

GENETIC ANALYSIS

The 100 patients in this study were from 98 family lines. Of 92 unrelated male patients, 13 (14%) had affected relatives in a previous generation, 12 (13%) had affected brothers only, and 67 (73%) were sporadic cases (patient numbers prefixed F, B, or S respectively in the appendix). Among the women, six were isolated cases and one was the daughter of a BMD patient who was also included in this study.

Table 1 summarises the gene mutations (deletions or duplications) found in each of the clinical groups. There were some subjects within every clinical group for whom no mutation could be found by Southern blot analysis. Deletions/duplications of the Xp21 gene were detected in 81-5% (75/92) of all male patients, 80-6% (54/67) occurring in isolated cases and 84% (21/25) in families with more than one affected member (data pooled for previous affected generations (n = 13) or brother pairs (n = 12) since, for these very small numbers, no statistical difference was detected). The proportion of sporadic cases versus multiple member families with such mutations was not significant. The ratio of mutations in the proximal and distal ‘hotspots’ was about 1:3 among the DMD patients but too few patients from multigeneration families were included in this study to compare this ratio in isolated versus familial cases. The 75 mutations detected included 15 deletions of a single exon (20%), 57 of more than one exon (70%), and three duplications of several exons (4%). Junctional fragments were found with 8% (6/75) of the deletions.

In the male patient groups the percentage with detectable mutations varied from 50% to 90%, but in the small group of female patients the only mutation detected (by dosage analysis of Southern blots) was in patient F94, the daughter of a Becker patient deleted for exons 45 to 48. No gross chromosomal rearrangements were found in any of the female patients. X chromosome inactivation studies were undertaken but the polymorphisms used in the differential methylation tests were uninformative for S96 and S97. Skewed X inactivation was found in twin S95 and patients S98 and S100, while random inactivation patterns within normal ranges were found in the young BMD carrier F94 using two independent methods.13

IMMUNOCYTOCHEMISTRY

The immunocytochemical (ICC) labelling patterns of dystrophin in individual biopsies were very similar with each of the monoclonal antibodies (MAbs) used. Scores for labelling with the rod and C-terminal MAb are given in the appendix. The value of using multiple antibodies is shown in fig 1A–C where a group 4 patient (S60) with a large deletion of exons 13 to 47 shows labelling only with the amino domain and C-terminal antibodies. The amino acids which form the antibody binding site of the rod MAb, Dy4/6D3, are missing. Unequivocal dystrophin labelling (ICC categories 3–5) was found in 24/41 (58%) of the group 1 and 2 DMD biopsies labelled with one or more of the antibodies. In general there was good agreement in the occasional fibres labelled with each MAb but there was not always complete concordance.

Among the male patients, the amount of ICC labelling (Materials and methods, labelling categories 1 to 8) increased according to clinical category so that BMD patients in group 5 showed most labelling (fig 2). Dystrophin labelling among the female patients was very variable but all had some negative fibres, which were found in groups in some cases (S95, S98, and S99). The remaining fibres either showed a continuous spectrum of labelling from near normal to near negative (a pattern found in the younger patients F94, S96, and S97), or there was a high percentage of fibres with normal labelling (60 to 85%) with smaller proportions of fibres with decreased (10 to 20%) dystrophin labelling (patterns found in the older and more mildly affected patients, S98, S99, and S100).

BLOTTING

As with the labelling on sections, the abundance of dystrophin labelling on blots gradually increased as the clinical condition improved from group 1 to group 5 (fig 3). Analysis of variance for all five groups indicates that there is a significant difference (p<0.001) and signi-

### Table 1

| Exons deleted or duplicated (dup) in different clinical groups (98 independent cases). |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Too young (n=7) | (n=21) | (n=20) | (n=6) | (n=4) | (n=31) | Females (n=6) |
| None (1) | None (2) | None (4) | None (3) | None (2) | None (3) | None (6) |
| 42-43 dup | 8-22 | *3-25 | *3 | 14-26 dup | *44 | *45-*47 (13) |
| 44 | J13-17 | J6-9 | 3-7 (2) | J5-16 du | *24-44 | *45-48 (7) |
| 45 | 20-29 | 3-16 du | 18-29 | *24-44 | *54 | *45-48 (4) |
| 45-49 | 22-41 | *10-13 | *3 | 14-26 dup | *65 | *45-48 (4) |
| 49-50 (2) | 36-43 | 44 (4) | 44 | *24-44 | *75 | *45-48 (4) |
| * | 45 | 45 | 45 | 45 | 45 | 45 |
| 45 | 45-52 | 45-52 | 45-52 | 45-52 | 45-52 | 45-52 |
| 45-54 | 45-54 | 45-54 | 45-54 | 45-54 | 45-54 | 45-54 |
| 45-57 | 45-57 | 45-57 | 45-57 | 45-57 | 45-57 | 45-57 |
| 48-50 | 48-50 | 48-50 | 48-50 | 48-50 | 48-50 | 48-50 |
| 50-51 | 50-51 | 50-51 | 50-51 | 50-51 | 50-51 | 50-51 |
| 52 (2) | 52 (2) | 52 (2) | 52 (2) | 52 (2) | 52 (2) | 52 (2) |
| #64-66 | #64-66 | #64-66 | #64-66 | #64-66 | #64-66 | #64-66 |
| 86% | 90% | 80% | 77% | 50% | 84% | 0% |

Numbers in parentheses indicate the number of subjects with the same mutation. Deletions marked with * are known to maintain the reading frame.

J = junctional fragment (deletion breakpoint close to, or in, an adjacent exon).
significant differences were also found when any two individual groups were compared (p<0.05 in all comparisons except that for groups 1 + 2 which was not significant). The appendix summarises the estimates of dystrophin abundance and size for all patients, and blots illustrating the dystrophin labelling in individual patients can be seen in part 2 of this investigation. In every case, we have only considered the largest size polypeptide detected in each patient sample lane.

One important difference between dystrophin labelling on blots and tissue sections was observed: while labelling on sections with the rod and C-terminal antibodies was virtually identical, labelling on blots was always less intense when the C-terminal MAb was used, which meant that labelling with the rod MAb had to be above a certain threshold before C-terminal labelling was detectable (fig 4). All estimates of dystrophin abundance were lower with the C-terminal MAb. Table 2 indicates this difference in 72 male patients (insufficient muscle was available from some patients to produce blots for labelling with more than one MAb). A significant difference (p<0.001) was found between the estimates of abundance using rod and C-terminal MAb within individual patients.

HISTOPATHOLOGY

Fig 5A shows that the variation in fibre size increased from group 1 to group 5 and analysis of variance was significant (p<0.001) for the five clinical groups. Nevertheless, the differences between any two individual groups were not significant, indicating that there was considerable overlap between the groups. Non-muscle tissue in biopsies is generally fibrous connective tissue (FCT) and fat. The percentage of FCT decreased across groups 1 to 5 (fig 5B) as clinical severity decreased and analysis of variance was highly significant (p<0.001). Significant differences between in-

Figure 1  Serial sections from a patient (S60) with a deletion of exons 13 to 47. Dystrophin labelling can be seen with the amino MAb Dy10/12B2 (A) and the C-terminal MAb Dy8/6C5 (C), but the deletion has removed the antibody binding site for the rod MAb Dy4/6D3 (B). (Indirect peroxidase.)

Figure 2  Immunocytochemical labelling with rod MAb Dy4/6D3. Each biopsy was scored for dystrophin labelling according to the categories described in Materials and methods (1 = negative, 8 = indistinguishable from normal).

Figure 3  Dystrophin abundance (mean, standard error) in different clinical groups estimated from densitometric analysis of blots labelled with the rod MAb Dy4/6D3. Note gradual increase with improved clinical condition. Raw data are in the appendix; group 1 n = 19, group 2 n = 19, group 3 n = 8, group 4 n = 4, group 5 n = 31. Patients with deletions which remove the antibody binding site, and DMD patient S39 (with atypical BMD-like dystrophin labelling), are not included.
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Figure 4  Dystrophin abundance estimated from blots labelled with the rod MAb Dy4/6D3 and the C-terminal MAB Dy8/6C5. A line is drawn at 45° to show where points would lie if the two parameters were identical. The points fall below the line which indicates that all estimates of dystrophin abundance were lower with the C-terminal MAb than with the rod MAb.

dividual groups were found only between groups 1 and 4 (p < 0.05) or 1 and 5 (p < 0.05).
In contrast to FCT, the percentage of fat showed extensive variation without regard to clinical group (fig 5C). Features which indicated acute histopathological changes (hyaline, necrotic, or regenerating fibres) were very variable, but showed a downward trend as clinical severity lessened from groups 1 to 5 (fig 5D).
Conversely, although some biopsies from each group showed features of chronic change (split or ring fibres), the highest scores were found in group 5 patients (fig 5E). Grouped (rather than isolated) fibres showing degeneration and regeneration were found in group 5 patients who presented to the clinic before 10 years of age. No obvious relationship was found between histopathological feature and dystrophin expression that did not also correlate with clinical severity or the age at which the biopsies were taken (range 7 weeks to 64 years).

Discussion

SELECTION OF PATIENTS

The patients in this study were selected for inclusion only if they had been examined in Newcastle, had undergone a muscle biopsy, and had provided a blood sample for DNA analysis. They represented the first 100 such patients that we were able to collect, and in fact constituted a reasonably typical sample of the clinical spectrum of patients with Xp21 muscular dystrophy seen in the Northern Region of England. The number of DMD and BMD patients was about equal and this was in agreement with new figures for disease prevalence (the number alive on a particular day) for the region. The number of female patients included was small and this reflects the number of such cases seen at our hospital. However, our cohort included a disproportionately large number of sporadic cases. In general, one third of cases of DMD (though not of BMD where the genetic fitness is not zero) would be expected to come from multigeneration families, one third from new mutations arising in the mother (producing brother pairs plus some isolated cases), and one third from new mutations arising in isolated males. In recent years, however, improved genetic counselling has reduced the number of affected boys born into families with a history of Duchenne muscular dystrophy. In our cohort 14% of males came from multigeneration families, 13% from brother pairs, and 73% were sporadic cases. This reflects the fact that biopsies were taken from all isolated cases whereas only the first presenting member in a family was usually subjected to this procedure. Many cases were excluded because we had biopsy material stored in the archive but no blood sample, patients having died or moved out of the area.

CREATINE KINASE

An apparent relationship was found between the level of serum creatine kinase (CK) activity at initial presentation to the hospital and the location of certain deleted exons. Relatively low CK values coincided with the cluster of deletions (45–47, 45–48, 45–49) found in group 5 Becker patients. However, high CK values were found in patients from all clinical categories (including group 5) who presented to the clinic before 7 years. Thus, as expected, the true relationship was between CK level and age at presentation rather than with the missing exons or ultimate clinical severity.

Table 2  Dystrophin abundance estimated from densitometric analysis of blots labelled with antibodies recognising epitopes in the mid rod and C-terminal domains.

<table>
<thead>
<tr>
<th>Clinical group</th>
<th>Rod domain</th>
<th>C-terminus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MAb Dy4/6D3</td>
<td>MAb Dy8/6C5</td>
</tr>
<tr>
<td></td>
<td>Range Mean (SD)</td>
<td>Range Mean (SD)</td>
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<td>35–90 51 (27)</td>
<td>8–53 23 (21)</td>
</tr>
<tr>
<td>5 n = 28</td>
<td>50–96 71 (15)</td>
<td>13–80 31 (15)</td>
</tr>
</tbody>
</table>

* Aliquots from the same muscle homogenates were used for both blots.

Patients S39 excluded as having atypical BMD-like dystrophin abundance.
Dystrophin abundance

In this study we found a significant difference between the apparent abundance of dystrophin on blots labelled with the rod MAb, Dy4/6D3, and with the C-terminal MAb, Dy8/6C5. However, labelling on sections was very similar with the rod and C-terminal MAb. It seems likely that the epitope recognised by Dy8/6C5 is very easily lost in the strenuous processing (homogenisation, boiling, centrifugation) required for Western blotting. In contrast, the preparation of unfixed frozen sections is a more gentle procedure and labelling with rod and C-terminal MAbs on sections was virtually identical, suggesting that the C-terminal MAb is not only reacting with a subpopulation of dystrophin molecules, such as might be generated by alternative splicing. The C-terminus is prone to degradation and the binding of a monoclonal antibody is even more vulnerable to slight changes in polypeptide conformation than a polyclonal antisera.

All the biopsy samples were labelled with the rod MAbs, Dy4/6D3, and the relationship between labelling on sections and labelling on blots was very close for this antibody. We therefore used blots labelled with this MAb for comparison with genotype and clinical phenotype. The precise techniques and reagents used to estimate the abundance of dystrophin from blots vary from laboratory to laboratory, and therefore absolute figures for abundance are not easily compared between centres. Nevertheless, the range of values for DMD and BMD within any centre might be considered. We detect dystrophin in a higher proportion of DMD patients than other laboratories and at a higher abundance. Nevertheless, the results of Hoff-
up to 17% of normal. Appropriately sized dystrophin was detected in two BMD patients with an abundance of 14 to 21% of normal, but results from a larger number of BMD cases would need to be reported before comparisons could usefully be made.

In this first part of our report we have described our methods, reviewed the patient cohort, and summarised the trends we observed across the clinical spectrum. In part 2 we will consider genotype and phenotype in more detail and report the correlations found within individual patients. In part 3 we use the data obtained from this study of patients with a wide range of disease severity to review which factors or types of analytical techniques appeared most useful for differential diagnosis and prognosis.

This study relied for a large part on the technical expertise of Keith Davison, Martin Barron, Liz O’Donnell, Gavin Falkous, Daisy Haggerty, and Marleen van Paassen for which we are extremely grateful. We also wish to thank Dr C Harwood for growing and harvesting the bacterial fusion proteins generously donated by Drs E Hoffman and L Kunkel, Drs K Davies, D Love, and R Cross, for the CF27 immunogen, and Dr D Mantle for the serum creatine kinase analysis. The financial support of the University of Newcastle upon Tyne Research Committee, the Muscular Dystrophy Group of Great Britain, the Wellcome Trust, the Medical Research Council, the Association Française contre les Myopathies (AFM), the Princess Beatrice Fund, the Dutch Prevention Fund, and the Netherlands Foundation of Medical Research (MEDIGON) is gratefully acknowledged.


Appendix Summary of data on the 100 patients in this study.

<table>
<thead>
<tr>
<th>Age</th>
<th>Patient group</th>
<th>Clinical group</th>
<th>Istm</th>
<th>Biopsy</th>
<th>Lomobility</th>
<th>Current</th>
<th>Deleted exons</th>
<th>Predicted dystrophin sizes [A] [B]</th>
<th>Observed dystrophin size</th>
<th>Dystrophin abundance (Rod/C-term)</th>
<th>ICC (Rod/C-term)</th>
<th>IQ</th>
<th>V</th>
<th>P</th>
<th>Full scores</th>
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<td>TY</td>
<td>S1</td>
<td>1:5</td>
<td>1:7</td>
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<td>5</td>
<td>4</td>
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<td>--</td>
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* = Predicted dystrophin sizes for patients with frame shifting deleions are calculated so [A] = size to premature stop codon, and [B] = size if reading frame immediately restored (see part 2).

--) data not available for patients with parentheses = data not certain, dup = exons duplicated rather than deleted. J = junctional fragment. ICC= immunocytotoxicity. V, P, = verbal IQ, performance IQ. ESN = classified as educationally subnormal. Patients FS5 and FS6, and patients P67 and P64 are related.