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1 **TITLE**

2 **Muscular dystrophy with marked Dysferlin deficiency is consistently caused by**
3 **primary dysferlin gene mutations**

4 Running title: Marked Dysferlin deficiency is primary

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1 **ABSTRACT**

2 Dysferlin is a 237-kDa transmembrane protein involved in calcium-mediated
3 sarcolemma resealing. Dysferlin gene mutations cause limb-girdle muscular dystrophy
4 (LGMD) 2B, Miyoshi myopathy (MM), and distal myopathy of the anterior tibialis.
5 Considering that a secondary Dysferlin reduction has also been described in other
6 myopathies, our original goal was to identify cases with a Dysferlin deficiency without
7 dysferlin gene mutations. The dysferlin gene is huge, composed of 55 exons that span
8 233,140 bp of genomic DNA. We performed a thorough mutation analysis in 65
9 LGMD/MM patients with $\leq 20\%$ Dysferlin. The screening was exhaustive, since we
10 sequenced both genomic DNA and cDNA. When required, we used other methods,
11 including real-time PCR, long PCR and array CGH.
12 In all patients we were able to recognize the primary involvement of the dysferlin gene.
13 We identified 38 novel mutation types. Some of these, such as a dysferlin gene
14 duplication, could have been missed by conventional screening strategies. Nonsense
15 mediated mRNA decay was evident in six cases, in three of which both alleles were
16 only detectable in the genomic DNA, but not in the mRNA. Among a wide spectrum of
17 novel gene defects, we found the first example of a “*nonstop*” mutation causing a
18 dysferlinopathy.
19 This study presents the first direct and conclusive evidence that an amount of Dysferlin
20 $\leq 20\%$ is pathogenic and always caused by primary dysferlin gene mutations. This
21 demonstrates the high specificity of a marked reduction of Dysferlin on western blot
22 and the value of a comprehensive molecular approach for LGMD2B/MM diagnosis.
23

- 1 **Keywords:** DYSF: dysferlin, LGMD: Limb-Girdle Muscular Dystrophy; MM: Miyoshi
- 2 Myopathy; NMD: Non sense Mediated mRNA Decay; CGH: Comparative Genomic
- 3 Hybridization
- 4

1 INTRODUCTION

2 Mutations in the dysferlin gene are responsible for three main dystrophic phenotypes:
3 limb-girdle muscular dystrophy type 2B (LGMD2B; MIM# 253601 ^{1,2}), Miyoshi
4 myopathy (MM; MIM# 254130 ²) and distal myopathy with anterior tibialis onset
5 (DMAT; MIM#606768 ³). During the course of the disease, the phenotypes show a
6 substantial amount of overlap with weakness extending from the proximal to distal
7 muscles and *vice versa* ^{4,5}. Even if clinical differences are reported, they may not be so
8 striking at the pathological level ⁶. A unique finding within the spectrum of muscular
9 dystrophies is that the majority of Dysferlin-deficient patients appear to have no initial
10 muscle weakness. Indeed, they often show a good performance at sport or have jobs that
11 require physical activity suggesting that exercise may be a disease-triggering factor ^{7,8}. A
12 secondary Dysferlin reduction has been also observed in calpain 3 (LGMD2A ⁹),
13 caveolin-3 (LGMD1C ¹⁰). A similar phenotype has been described also in patients with
14 anoctamin 5 gene (LGMD2L ¹¹) mutations.

15 The dysferlin gene is huge and routine mutation detection is a long and expensive
16 process. In addition, there are no prevalent mutations, but, instead, 415 different allelic
17 variants have been identified thus far (www.dmd.nl). The cost-effectiveness of a
18 dysferlin gene mutation screening using DHPLC approaches has been reported in large
19 groups of patients with the identification of many mutations, so validating the efficacy
20 of genomic mutational screening for routine diagnosis ¹².

21 Most authors have been able to identify the majority of dysferlin gene mutations (but
22 not all), when Dysferlin was absent from the muscle ^{13,14}. Several mutational screenings
23 have been reported, many of them based on isolated cases/families and the identification
24 of private mutations ¹⁵⁻²². The biggest cohort was analyzed by Krahn *et al.* ¹², composed

1 of 134 patients, including a group that had previously been described¹⁸. The authors
2 identified at least one pathological allele in 119/134 patients (88%). The success rate
3 was higher than in other LGMD cases²³, but not all cases were solved, indicating either
4 that other genes causing secondary defects of Dysferlin are rarer, or that the mutation
5 analyses have been inaccurate in that they have missed some of the defects.

6 Our original aim was to focus on LGMD cases with a marked Dysferlin reduction and
7 no mutation. To achieve this objective we performed a complete mutation analysis of
8 the dysferlin gene in a group of 65 LGMD/MM patients with a marked reduction or
9 absence of the protein detected by western blot. We used all available screening
10 methods starting from genomic DNA and mRNA. Here we show the high specificity of
11 the absence or marked reduction of dysferlin expression on muscle biopsy and the high
12 specificity and sensitivity of a comprehensive molecular approach in the diagnosis of
13 LGMD2B/MM.

14

1 **MATERIALS AND METHODS**

2 **Patient recruitment**

3 The patients were between 26 and 77 years of age. They had serum levels of creatine
4 kinase between 1,200 U/L and 8,000 U/L (with an average value of 3,507 U/L). These
5 patients showed an absence or marked reduction (5-20% of the normal amount) of
6 Dysferlin in their skeletal muscle. Ten out of 65 were affected by Miyoshi myopathy,
7 while the other 55 had an LGMD2B phenotype. In our study we analysed 8 pairs of
8 siblings. Only three of these patients are wheelchair-confined in accordance with the
9 slow progression rate of the dysferlinopathies. In particular, the age at which they began
10 to use a wheelchair was very variable (22, 39 and 49 years of age respectively), again in
11 accordance with the phenotypical variability of the dysferlinopathies^{5,24,25}.

12 Biological samples were obtained from 65 patients (61 of which Italians) from different
13 centres: Naples (16/65), Rome (5/65), Genoa (8/65), Padua (31/65), Ankara (Turkey)
14 (3/65) and Buenos Aires (Argentina) (1/65). For all these patients genomic DNA and/or
15 mRNA from blood samples or muscle biopsies were available.

16

17 **Muscle biopsy**

18 At the time of diagnosis, an open biopsy from the quadriceps femoris muscle was
19 obtained under local anesthesia after written informed consent.

20 Muscle biopsy specimens were frozen in isopentane, cooled in liquid nitrogen and
21 stored at -80°C until processed.

22

23 **Multiple Western blot analysis of muscle proteins and quantification**

1 Western blot (WB) analysis and detailed protein analyses were performed as previously
2 described²⁶ using a mixture of monoclonal antibodies against Calpain-3 (Calp12A2,
3 diluted 1:800), alpha-Sarcoglycan (diluted 1:300), beta-Sarcoglycan (diluted 1:300),
4 Dystrophin (Dys-2, diluted 1:1000), and Dysferlin (Hamlet, diluted 1:1000), all
5 purchased from Novocastra (UK). In brief, the quantity of muscle proteins in the
6 samples from the controls and patients was determined by densitometry using ImageJ
7 software v.1.34n and normalised to the amount of tissue loaded in each lane, using the
8 skeletal myosin bands in the post-transfer Coomassie blue-stained gels. The values in
9 each patient were expressed as percentages of the mean of the controls.

10

11 **PCR conditions from gDNA**

12 The dysferlin gene (NM_001130987.1, 55 coding exons) was amplified by PCR from
13 genomic DNA. All the exons and flanking intron sequences were amplified using
14 specific primer pairs (Supplementary Table S1). In a final volume of 25 μ l, 60-75ng of
15 genomic DNA were combined with 0.6 μ M of each primer, 0.14mM dNTPs, buffer
16 LB1X (20mM Tris, 10mM Hepes, 2.5mM magnesium sulphate, 10mM potassium
17 chloride, 10mM ammonium sulphate) or buffer LC1X (20mM Tris, 10mM Hepes,
18 2.5mM magnesium sulphate, 20mM ammonium sulphate, 5% glycerol), and 0.9 U of
19 AmpliTaq-Gold (Perkin Elmer, Massachusetts, USA). After polymerase activation 7
20 min at 95°C, reactions were then carried out for 30 seconds at 95 °C, 1 minute at T_m
21 (see Supplementary Table S1) and 1 minute at 68 °C, for 30 cycles.

22

23 **DHPLC Analysis**

1 We performed comparative mutation scanning to select amplicons for aberrant DHPLC
2 profiles not shared by the normal controls. DHPLC analysis was performed on a WAVE
3 DNA fragment analysis system (Transgenomic Inc., San Jose, CA) equipped with a
4 DNASep column (3,500 High Throughput [HT]) employing a UV-C scanner to detect
5 eluted DNA ²⁷.

6

7 **mRNA extraction and cDNA preparation**

8 We used a TRIzol® reagent (Invitrogen Carlsbad, California) according to the
9 manufacturer's instructions to extract RNA from the muscle biopsies and the
10 PAXgene™ Blood RNA Kit (Qiagen, Hilden, Germany) to extract RNA from the
11 blood²⁸.

12 The retrotranscription reaction was performed using 2µg of total mRNA according to
13 the SuperScript® III kit (Invitrogen Carlsbad, California).

14

15 **RT-PCR and long PCR**

16 We amplified the dysferlin cDNA in 13 overlapping fragments (Supplementary Table
17 S2). The reaction was performed in a final volume of 25µl using: 1µl of cDNA, 1µM of
18 each primer, 0.8mM of dNTPs, Buffer JD1X ²⁹, 0.5U of LA-Taq DNA polymerase
19 (Takara BIO Inc.) and 1U.I. of Pfu polymerase (Stratagene). Thermocycling was then
20 carried out for 30 seconds at 94 °C, 1.30 minute at T_m (see Supplementary Table S2)
21 and 2 minute at 68 °C, for 30 cycles.

22 Fragments were recovered from agarose gels by using the Mini Elute™ Gel Extraction
23 Kit (Qiagen, Hilden, Germany) and then sequenced.

24

1 **Sequencing**

2 BigDye® Terminator sequencing chemistry and ABI3130XL automatic DNA sequencer
3 (Applied Biosystems, Foster City, CA) were used. Each nucleotide change was verified
4 by reverse sequencing and, in addition, by the sequencing of an overlapping PCR
5 product obtained with different primers. Mutations were numbered based on protein
6 (GenBank NP_003485) and cDNA sequence (GenBank NM_003494). Nucleotides
7 were numbered according to international recommendations³⁰⁻³²

8

9 **Array CGH**

10 A custom array CGH (MotorChip 2.0) was developed using the Agilent 8X60K format
11 (SurePrint G3 arrays). All dysferlin exons, both the 5'- and 3'-UTR, 2,000 bp at the
12 5' end of the gene (covering the dysferlin promoter) were included. Probes were
13 designed based on the exon and flanking intron sequences. Array CGH results were
14 confirmed by independent assays, such as real time PCR, long PCR and MLPA.

15

16 **Bioinformatic software**

17 Splice View software was useful to verify the effect of intronic variants on mRNA
18 splicing (<http://bioinfo.itb.cnr.it/oriel/splice-view.html>). Conservation analysis was
19 performed using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). To
20 assess intronic and exonic mutations leading to splicing defects, the Human Splicing
21 Finder website (<http://www.umd.be/SSF/>) was consulted³³.

22

1 **RESULTS**

2 To select patients with a Dysferlin deficiency from a heterogeneous population of
3 patients affected with an unclassified form of LGMD or MM, we previously analyzed
4 muscle samples by a multiple WB. A group of 65 patients that showed a marked
5 reduction or absence of Dysferlin were included in the study (fig. 1A, B). We excluded
6 cases having more than 20% Dysferlin using a quantitative WB assay²⁶. The average
7 Dysferlin level was $5\% \pm 5$. We combined different screening methods to identify the
8 causative alleles (fig. 1C).

9
10 **From genomic DNA - DHPLC**

11 Despite the incomplete DHPLC sensitivity and the noise of the variants and
12 polymorphisms, this first step was chosen for its cost-effectiveness³⁴. We screened by
13 DHPLC and targeted a sequencing of all the 55 exons and flanking introns from the
14 genomic DNA. DHPLC analysis was performed on a first group of 52 patients. We
15 identified 47 causative mutations, 28 of which were new (Supplementary Table S3). A
16 full molecular diagnosis (both alleles) was provided for 35 out of 52 patients (67%).

17
18 **mRNA analysis**

19 We next performed mRNA analysis in cases without mutations or with ambiguities (one
20 allele, new mutations, possible splice defects, etc.). We first confirmed on cDNA the
21 splice mutations. We also extended the analysis to an additional group of 13 patients.
22 Two sources of mRNA were considered: 1) skeletal muscle that, when available, is the
23 first choice for the expression level and canonical splicing of muscle dysferlin; 2)
24 leukocytes that are easy to collect and representative of non muscle dysferlin, with some

1 differences in the alternative splicing of exons 5a, 17 or 40a^{35,36}. For this analysis we
2 performed 16 muscle biopsies and, when not possible, we collected blood samples. The
3 cDNA was amplified by PCR in 13 overlapping fragments and then sequenced (fig.S1).
4 We identified 15 mutations, eight of which were novel. mRNA analysis was useful to
5 demonstrate the effect of two intronic variations on RNA splicing (fig. 2A-B). Patient
6 X311 carrying the homozygous mutation 906+4A>G showed a smaller amplification
7 product of fragment 3 (including approximately exons 7 to 12) of the dysferlin cDNA
8 compared to the control (fig. 2A). Direct sequencing showed that exon 9 was skipped
9 (fig. 2A'). At the protein level, exon-9 skipping causes the loss of a part of the C2B
10 domain, important for the protein function. Therefore, this variation should be
11 considered as pathological.

12 The transversion 1639-6T>A, found in a homozygosity in patient X389, inactivated the
13 donor splice site (with the reactivation of a new donor site in intron 18) resulting in
14 retention of 4bp from intron 18 sequence (fig. 2B) and a frame-shift (Supplementary
15 Table S3).

16 Patient X546 (classified as having severe LGMD2B) showed < 5% Dysferlin.
17 Molecular analysis confirmed the presence of a frameshift mutation, 1-bp deletion
18 (g.2077delC) on exon22 (fig. 2C), and the heterozygous deletion of the entire exon 17
19 (fig. 2D). Exon 17 skipping did not affect the open reading frame, but the amino acid at
20 the exon junction changed (GAG>GTG, g.1481_1522del, p.E494V+ex17skipping). The
21 alternative splicing of the exon 17 had previously been identified and characterized as
22 blood cell isoform³⁶, but it had never been detected in muscle.

23

24 **DNA re-sequencing**

1 All-exon and flanking intron resequencing was used to confirm all the mutations. We
2 identified three additional mutations, the diagnosis being completed in 58/65 patients
3 (Table 1). Surprisingly, six apparently homozygous alleles were not confirmed by the
4 DNA analysis. These alleles were heterozygous and for 3 out of 6 patients a second
5 nonsense mutation was identified in other regions of the gene. This can be explained by
6 the nonsense mediated decay (NMD) of the mRNA¹⁶ (fig. 2E). For the other three
7 patients with NMD the promoter region was not mutated³⁷.

8

9 **Array CGH, real time PCR and long PCR**

10 In all cases with an incomplete gene testing (0 or 1 mutated allele) we performed a CGH
11 array to identify possible heterozygous intragenic rearrangements, such as deletions or
12 duplications³⁸ (Table 1). We used a minimum of three probes per exon plus promoter
13 probes covering a genomic region of 220kb using a total of 196 probes. We identified a
14 heterozygous duplication involving the 5' end of the gene from exon 1 to 22 (3484), and
15 a heterozygous deletion (X583). In addition, we performed a long PCR on the mRNA
16 and/ or gDNA using primers located at distant positions in the transcript to confirm the
17 alterations.

18

19 **Mutation spectrum**

20 The exhaustive molecular analysis of the dysferlin gene led to the identification of 65
21 different mutations, 38 of which (60%) had not previously been described
22 (Supplementary Table S3 and fig.3). Thirty-one patients showed homozygous alleles
23 (47.7%), most confirmed by segregation studies. Mutation scanning methods used in
24 our study led to the identification of 19 additional novel non-pathological variants

1 (Supplementary Table S4). The present study confirmed the absence of a mutational
2 hot-spot region as well as the spreading of mutations along the entire gene. We detected
3 9 (14%) nonsense mutations, 28 (43%) missense mutations, 5 (8%) splicing mutations,
4 20 (30.5%) frame shift mutations, one nonstop mutation (1.5%), and two large genomic
5 rearrangements (3%) (Table 2).

6 For the novel mutations their absence was demonstrated in > 1,000 control
7 chromosomes from healthy individuals of matched ethnic origin. In all 10 patients with
8 10-20% of Dysferlin we always found at least one missense mutation (100%), while
9 among 24 patients with 0% Dysferlin a missense mutation was only found in 7 cases
10 (29%).

1 **DISCUSSION**

2 The present study demonstrates that the marked reduction of Dysferlin observed in
3 LGMD/MM patients is not genetically heterogeneous. Given the location of Dysferlin,
4 close to the muscle membrane, its interactions with other proteins, and cumulative data
5 about the existence of secondary dysferlinopathies, this is surprising, because in other
6 membrane complexes, such as sarcoglycans, a marked secondary reduction of each
7 component is common. A Dysferlin reduction has been observed in primary calpain 3
8 deficiency (LGMD2A ⁹) or Caveolin 3 deficiency (LGMD1C ¹⁰). An LGMD/MM
9 phenotype has also been observed in patients carrying mutations in the Anoctamin 5
10 gene (LGMD2L ¹¹).

11 Our results derive from an exhaustive analysis of RNA and DNA from 65 patients
12 having a severe Dysferlin reduction. In theory, it has been claimed that a DNA analysis
13 is necessary to diagnose a primary dysferlinopathy ³⁹. In practice, however, it is not
14 easy. The dysferlin gene is huge and composed of 55 exons. It spans 233,140bp of
15 genomic DNA and generates a 6.9 kb-wide transcript. In our mutation screening
16 flowchart (fig.1), the DNA analysis was first carried out by a DHPLC of all exons and
17 flanking introns. DHPLC is cheaper, but it can give false-negative/positive results and
18 therefore it can only have a screening value ⁴⁰. A second problem with DHPLC consists
19 in the huge number of polymorphisms and variants that are present in this gene
20 (Supplementary Table S4). These are located in all exons and confound an
21 interpretation of the results with many heteroduplex shifts per patient. We therefore
22 used a second technique based on the sequence analysis of the dysferlin cDNA. When a
23 muscle biopsy was not available, we were able to analyze the patients' mRNA from
24 blood, since Dysferlin is highly expressed in the monocytes. This method ^{28,35} is less

1 invasive and can provide an adequate amount of mRNA: the analysis helped us to
2 understand the pathogenic role of the two intronic variants identified by DNA analysis,
3 both leading to an alteration of the splicing mechanism (fig. 2A-B).

4 Despite the larger number of cases identified by mRNA analysis, this method alone can
5 be faulty when the mutated allele is not expressed. We showed that the mechanism of
6 *nonsense-mediated mRNA decay* (NMD) also occurred in dysferlinopathy¹⁶. In six out
7 of 65 patients (fig. 2D) we identified a homozygous mutation from cDNA, a mutation
8 which was heterozygous from gDNA. By direct sequencing of gDNA we identified in
9 3/6 (X584, X674, X676) an additional frameshift mutation missed by DHPLC. For the
10 three other patients (X267, X268, X675), we failed to identify the primary cause of the
11 missing mRNA expression of the second allele, but the NMD anyhow confirmed the
12 primary involvement of the dysferlin gene.

13 This confirms that mRNA analysis alone can be faulty, since true homozygote patients
14 cannot be distinguished from compound heterozygote patients with important
15 consequences in respect of genetic counselling.

16 Thirdly, we resequenced all the relevant genomic regions and three additional mutations
17 were found. Furthermore, we used three additional methods: long PCR, real time PCR
18 and array CGH.

19 Particularly noteworthy was the first evidence of a *non stop* mutation as a new
20 pathological mechanism involved in the dysferlinopathies. Patient X295 carries a
21 homozygous 8-bp (g.6233_6240del, p. P2078fsNON STOP) deletion that was identified
22 in exon 55 of the dysferlin gene. The deletion led to a frameshift in the reading frame
23 with the loss of the stop codon (fig. 4A, B). We supposed that the new reading frame
24 could give rise to the translation of 97 additional amino acids through the 3'end of the

1 mRNA. The patient showed a residual expression of <10% of larger sized Dysferlin in
2 the skeletal muscle (fig. 4C). We hypothesize that the mutation could cause a
3 *mechanism of nonstop mRNA decay*. Indeed, it was demonstrated that in the eukaryotes
4 there is a mechanism of degradation of mRNA lacking the stop codon⁴¹⁻⁴⁴. *Non stop*
5 mutation has previously been identified in the ACTA1 gene⁴⁵.

6 Many groups have questioned the value of protein analysis in carrying out a correct
7 diagnosis. Fanin *et al.*²⁶ observed that the levels of Dysferlin were reduced to 50% of
8 those of the controls in the carriers of LGMD2B. They showed that a reduction of 50%
9 indicated both familial and isolated LGMD2B heterozygotes, and suggested the use of
10 Dysferlin protein testing to select muscle biopsies from suspected carriers for a
11 subsequent mutation analysis²⁶.

12 Our data support the dysferlin gene as the unique cause of Dysferlin deficiency between
13 0% and 20% by WB analysis. Although for three patients (3484, 4132 and X147) the
14 second allele was not identified, this was only due to incomplete testing for insufficient
15 DNA. However, this does not affect the main conclusions of the study, because these
16 patients show sure causative alleles (frame-shift/duplication) that cannot be
17 coincidental.

18 This marked reduction is necessary to affect muscle membrane repair. We cannot
19 exclude the presence of other functional mutations, but a direct proof of pathogenicity is
20 always required, since the dysferlin gene shows a large number of variants and
21 polymorphisms that can be misleading. In these cases, the possible lack of mutations in
22 the dysferlin gene may be due to incomplete genetic testing.

23 The results obtained in this present paper have an immediate diagnostic application: a
24 Dysferlin reduction to 20% (that can also be measured from blood monocytes^{28,35}) can

1 be used to identify LGMD2B with 100% accuracy. In the case of LGMD2B this
2 observation is noteworthy. When a rapid Dysferlin blood testing will be available,
3 important decisions will derive, such as to avoid steroids (that are
4 ineffective/deleterious in LGMD2B in contrast with other forms of muscular dystrophy)
5 and any distressing sport activities in children^{7,46}.

6 Although, it is generally agreed that an extensive molecular analysis has a high cost, a
7 precise determination of the DYSF gene is, however, particularly important from a
8 diagnostic/counselling perspective and in view of the development of a future
9 therapeutic strategy. A successful recognition of all the mutations demonstrates the
10 power of a combined diagnostic strategy. More importantly, a complete genetic testing
11 should be applied to all other LGMD cases⁴⁷.

12

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23

24 **Conflict of interest**

1 The authors declare that they have no conflict of interest.

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TITLES AND LEGENDS TO FIGURES

Figure.1: Mutational Scanning. **A.** The picture shows the western blot on muscle lysate from patients (1, 2) and control (C). As observed, patient 1 showed a complete absence of the dysferlin-specific band, while sample 2 shows a residual expression (5%) of dysferlin protein, compared to the control (as indicated by the arrow). **B.** An exemplary image of a multiple western blot assay used in this study to screen and select the patients for further molecular analyses (samples 1 and 2 show a complete dysferlin deficiency). For both A and B, the skeletal myosin bands in the post transfer Coomassie blue staining gels were used to normalize the amount of loaded protein. **C.** The flow chart shows the general design of the mutation analysis.

Figure 2 Elusive/peculiar mutations: **A:** Exon 9 skipping in a patient with the variation 906+4A>G in homozygous status. RT-PCR products between DYSF exons 7 and 12 showing the aberrantly-spliced transcript. **A:** Graphical presentation of the sequence composition of the wild-type transcript and the aberrantly-spliced transcript with the exon 9 skipping. **B.** Partial intron 18 retention in a patient with the variation 1639-6T>A in homozygous status. RT-PCR products sequencing between DYSF exons 12 and 19 showing a 4 bp retention of the intron 18, which is predicted to result in a truncated protein. **C-D:** Analysis of the cDNA sequence of patient X546 for C fragment 5 (including exon 22) and D fragment 4 (including exon 17). Both mutations produce a frameshift. **E:** Sequence analysis performed on specific fragments of dysferlin obtained from cDNA and DNA. The same mutation is shown for both cDNA and DNA. The mutated base is highlighted in blue. All the patients showed the mutation in homozygous status on cDNA and heterozygous status on DNA.

Figure 3: Mutation spectrum. The picture shows the position of all the mutations identified in this study. Δ identifies missense mutations; \circ identifies the frameshift (del/ins) mutation; \diamond identifies the mutations affecting the splicing mechanism; \bullet identifies the non sense mutation

Figure 4: Non stop mutation. A-B: Analysis of DNA sequence obtained from a control (A) and patient (B). The mutated base is highlighted in blue. The sequence of exon 55 shows a homozygous deletion of 8bp (g. 6233_6240del; p. Pro2078LeufsNON STOP). The frameshift skips the stop codon and produces hypothetically the translation of 97 additional amino acids. Both nucleotide and translated sequences are reported for both samples. **C:** The picture shows the western blot on muscle lysate from a patient (X295) and control (C). As observed, patient X295 showed a residual expression (<10%) of larger sized Dysferlin, compared to the control (as indicated by the arrow)

Table 1: Number of causative alleles identified by the different techniques

mutations	DHPLC DNA	mRNA sequencing	gDNA sequencing	Array CGH Real Time/long PCR
0 alleles	5	0	0	0
1 allele	12	8	5	5
2 alleles	35	18	4	0
Samples examined	52	26	9	5

Table 2: Classification of all mutation types identified (Het: heterozygote, Hom: homozygote). Numbers indicate how many mutation types were discovered

<i>Class of Mutation</i>	<i>Mutations (%)</i>	<i>Status</i>	
		<i>Het</i>	<i>Hom</i>
Missense	28 (43%)	27	12
Nonsense	9 (14%)	9	5
Splice site	5 (8%)	2	5
Frameshift	20 (30.5%)	24	8
Non-Stop	1 (1.5%)	0	1
Genomic Rearrangements	2 (3%)	2	0







