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## Muscular dystrophy with marked Dysferlin deficiency is consistently caused by primary dysferlin gene mutations

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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 <sup>1,2</sup>), Miyoshi  
4 myopathy (MM; MIM# 254130 <sup>2</sup>) and distal myopathy with anterior tibialis onset  
5 (DMAT; MIM#606768 <sup>3</sup>). 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* <sup>4,5</sup>. Even if clinical differences are reported, they may not be so  
8 striking at the pathological level <sup>6</sup>. 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 <sup>7,8</sup>. A  
12 secondary Dysferlin reduction has been also observed in calpain 3 (LGMD2A <sup>9</sup>),  
13 caveolin-3 (LGMD1C <sup>10</sup>). A similar phenotype has been described also in patients with  
14 anoctamin 5 gene (LGMD2L <sup>11</sup>) 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](http://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 <sup>12</sup>.

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 <sup>13,14</sup>. Several mutational screenings  
23 have been reported, many of them based on isolated cases/families and the identification  
24 of private mutations <sup>15-22</sup>. The biggest cohort was analyzed by Krahn *et al.* <sup>12</sup>, composed

1 of 134 patients, including a group that had previously been described<sup>18</sup>. The authors  
2 identified at least one pathological allele in 119/134 patients (88%). The success rate  
3 was higher than in other LGMD cases<sup>23</sup>, 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<sup>5,24,25</sup>.

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  $-80^{\circ}\text{C}$  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 described<sup>26</sup> 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 µl, 60-75ng of  
15 genomic DNA were combined with 0.6 µM 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<sub>m</sub>  
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 <sup>27</sup>.

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 blood<sup>28</sup>.

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 <sup>29</sup>, 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<sub>m</sub> (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 recommendations<sup>30-32</sup>

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 consulted<sup>33</sup>.

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 assay<sup>26</sup>. 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-effectiveness<sup>34</sup>. 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<sup>35,36</sup>. 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 isoform<sup>36</sup>, 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 mRNA<sup>16</sup> (fig. 2E). For the other three  
7 patients with NMD the promoter region was not mutated<sup>37</sup>.

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 duplications<sup>38</sup> (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 <sup>9</sup>) or Caveolin 3 deficiency (LGMD1C <sup>10</sup>). An LGMD/MM  
9 phenotype has also been observed in patients carrying mutations in the Anoctamin 5  
10 gene (LGMD2L <sup>11</sup>).

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 <sup>39</sup>. 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 <sup>40</sup>. 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 <sup>28,35</sup> 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 dysferlinopathy<sup>16</sup>. 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 codon<sup>41-44</sup>. *Non stop*  
5 mutation has previously been identified in the ACTA1 gene<sup>45</sup>.

6 Many groups have questioned the value of protein analysis in carrying out a correct  
7 diagnosis. Fanin *et al.*<sup>26</sup> 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 analysis<sup>26</sup>.

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<sup>28,35</sup>) 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<sup>7,46</sup>.

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 cases<sup>47</sup>.

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.  $\Delta$  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**

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







