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Recessive *RYR1* mutations cause unusual congenital myopathy with prominent nuclear internalization and large areas of myofibrillar disorganization

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Aims: To report the clinical, pathological and genetic findings in a group of patients with a previously not described phenotype of congenital myopathy due to recessive mutations in the gene encoding the type 1 muscle ryanodine receptor channel (RYR1). **Methods:** Seven unrelated patients shared a predominant axial and proximal weakness of varying severity, with onset during the neonatal period, associated with bilateral ptosis and ophthalmoparesis, and unusual muscle biopsy features at light and electron microscopic levels. **Results:** Muscle biopsy histochemistry revealed a peculiar morphological pattern characterized by numerous internalized myonuclei in up to 51% of fibres and large areas of myofibrillar disorganization with undefined borders. Ultrastructurally, such areas frequently occupied the whole myofibre cross section and extended to a moderate number of sarcomeres in length. Molecular genetic investigations identified recessive mutations in the ryanodine receptor (*RYR1*) gene in six compound heterozygous patients and one homozygous patient. Nine mutations are novel and four have already been reported either as pathogenic recessive mutations or as changes affecting a residue associated

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JAB and NM contributed equally to this work.

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with dominant malignant hyperthermia susceptibility. Only two mutations were located in the C-terminal transmembrane domain whereas the others were distributed throughout the cytoplasmic region of RyR1. **Conclusion:** Our data enlarge the spectrum of *RYR1* mutations and highlight their clinical and morphological heterogeneity. A congenital myopathy featuring ptosis and external ophthalmoplegia, concomitant with the novel histopathological phenotype showing fibres with large, poorly delimited areas of myofibrillar disorganization and internal nuclei, is highly suggestive of an *RYR1*-related congenital myopathy.

Keywords: congenital myopathy, myofibrillar disorganization, nuclear internalization, recessive mutations, *RYR1* gene

Introduction

The *RYR1* gene (OMIM 180901) encodes the ryanodine receptor 1, a Ca^{2+} channel expressed on sarcoplasmic reticulum membranes at the triad of skeletal muscle fibres. RyR1 mediates the release of Ca^{2+} from intracellular pool in response to nerve stimulation and then plays a crucial role in excitation–contraction coupling [1]. Mutations of the *RYR1* gene cause well-defined forms of congenital myopathies, that is, central core disease (CCD; OMIM 117000) and malignant hyperthermia susceptibility (MHS; OMIM 145600), an autosomal dominant pharmacogenetic disease. This gene is also implicated in some cases identified as multi-minicore disease (MmD; OMIM 602771). The *RYR1* mutations associated with CCD are usually dominant but recessive inheritance has also been reported, whereas cases identified as MmD are exclusively linked to recessive mutations [2–7] and recently in patients with fibre type disproportion as their only pathological feature. [8] Classically in the *RYR1* sequence, three hot-spots are considered, two in the large hydrophilic domain of RyR1 and one in the C-terminal hydrophobic domain. Most of the heterozygous dominant CCD mutations are mapped to the C-terminal domain, whereas the recessive CCD and MmD mutations are more extensively distributed along the *RYR1* sequence. Additionally, a heterozygous *de novo RYR1* mutation in the C-terminal region of the protein has been found in a 16-year-old female patient initially diagnosed with centronuclear myopathy (CNM) with 'core-like' lesions and central nuclei in up to 50% of fibres in the muscle biopsy [9], and a heterozygous *de novo RYR1* mutation in the N-terminal domain has been found in a patient presented with King-Denborough syndrome and MHS [10].

In *RYR1*-related congenital myopathies, the histological phenotype varies widely. It comprises central and eccentric cores, unique and multiple, structured and unstructured, well-delimited cores spanning the entire fibre length or poorly defined cores that spread only a few sarcomeres, and occasionally a variable degree of sarcomeric disorganization [2,11–13]. These structural abnormalities are sometimes associated with an increased number of internal myonuclei (up to 30% of the fibres) and variable degrees of fibrous and adipose tissue replacement [6,14,15]. There also exist biopsies without major alterations showing only a type I fibre predominance or uniformity [16]. Moreover, a histopathological continuum has been suggested linking the diverse *RYR1*-related core myopathies [17–20]. On the other hand, centronuclear myopathies (CNM; OMIM 310400, 160150 and 255200), comprise X-linked recessive, autosomal dominant and autosomal recessive forms, associated, respectively, with myotubularin 1 (*MTM1*), dynamin 2 (*DNM2*) and amphiphysin 2 (*BIN1*) genes [21–23]. The histopathological presentation of these distinct forms of CNM has been well established [24]; so far, neither cores nor minicores have been described in such genetically determined CNM forms.

Here we report clinical, histological and molecular characterization of seven patients initially diagnosed with CNM due to the significantly high number of fibres with internalized nuclei (up to 51% of the fibres). However, the key histopathological feature that led us to screen *RYR1* gene for mutations was the invariable presence of large areas of sarcomeric disorganization in the muscle fibres, despite the number and location of internalized nuclei. Thus, *RYR1* recessive mutations were found in every patient of the series demonstrating that this peculiar disorder should be classified as a form of *RYR1*-related congenital myopathy.

Methods

Patients

We retrospectively reviewed the clinical and histological data of patients with an original diagnosis of CNM

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without *DNM2* mutations. We identified seven unrelated patients (five women and two men) (Table 1) who shared the same morphological findings in the muscle biopsy (see *Results*). This study was authorized by the ethical committee of Pitié-Salpêtrière Hospital (CCPPRB) and the Direction de Recherché Clinique of the Assistance Publique, Hôspitaux de Paris.

Histopathological studies

Skeletal muscle biopsies were obtained from all patients. Age of patient and the biopsied muscles were indicated in Table 1. Histological, histoenzymological and electron microscopic analyses were performed as previously described [25]. Ultrastructural studies were performed in all patients except patient 2. The number of fibres with nuclear centralization (that is, myonuclei in the geometric centre of the fibre) and with nuclear internalization (that is, myonuclei underneath the sarcolemma anywhere within the cytoplasm) were counted in a minimum of 200 adjacent muscle fibres. In each biopsy, the diameter of type 1 and type 2 fibres stained with myosin adenosine triphosphatase (ATPase) 9.4 was measured manually on digital pictures in at least 120 fibres using ImageJ 1.40g® (NIH, Washington, USA).

Molecular genetics studies

Informed consent for genetic analysis was obtained from each patient and their families. *RYR1* mutation screening was performed on cDNA obtained after reverse transcription of total RNA extracted from muscle specimens as previously described [2]. The cDNA was amplified in overlapping fragments. Sequencing reactions were analysed on an ABI 3130 DNA Analyzer (Life Technologies, Foster City, CA, USA). The presence of the mutations identified in transcripts was confirmed in genomic DNA by direct sequencing of the corresponding exon and intron–exon junctions. None of the novel variants was found in 200 chromosomes from the general population. To evaluate the consequences of the c.8692+131G>A mutation at the transcription level, cDNA fragments encompassing exons 56 and 57 were amplified and cloned using the TOPO TA Cloning® Kit (Invitrogen, Carlsbad, CA,USA). After transformation into One Shot Competent DH5α™-T1^{R} cells (Invitrogen), colonies containing the recombinant plasmids were identified by PCR using *RYR1* specific primers, and the cDNA inserts were sequenced.

Protein analysis

To analyse the expression of RyR1, thin slices of frozen muscle biopsies from patients 1 and 6 were homogenized in Hepes 20 mM (pH 7.4), sucrose 200 mM, $CaCl_2$ 0.4 mM, Complete Protease Inhibitor® cocktail (Roche, Meylan, France). The amount of RyR1 present in each muscle sample was determined by quantitative Western blot analysis using antibodies directed against RyR1 as described previously [26]. Signals were detected using a chemiluminescent horseradish peroxidase (HRP) substrate and quantified using a ChemiDoc XRS apparatus (Biorad, Hercules, CA, USA) and the Quantity 1 software (Biorad).

Results

Patients

The parents of the seven patients were clinically unaffected. All patients except patient 6 were born from non-consanguineous families. Patient 1 was the second daughter of a family with two affected and two non-affected children, and her eldest affected sister died at 5 months of age due to severe respiratory impairment and weakness; all the other patients were sporadic cases. Prenatal symptoms were noted only in patient 2 with reduced foetal movements. At birth, the seven patients showed generalized hypotonia, poor spontaneous movements and amyotrophy, together with weak suction and swallowing difficulties. Motor development was delayed in all patients. Poor head control was noted in patients 1 and 2, who required support to sit or walk. Since early childhood, patients showed difficulties in rising up from the floor, climbing stairs and running. Patients progressively improved their motor capabilities and have acquired independent ambulation with the exception of patient 1. Significant facial involvement (hypomimia, open mouth, facial diplegia and elongated facies) was observed particularly in patients 1 and 2, and at a moderate level in the other patients. All patients showed some degrees of ocular involvement consisting of either ptosis or ophthalmoparesis with limited upward gaze or incomplete eyelid closure. Serum creatine kinase levels were normal or slightly increased. A computed tomography (CT) scan performed to patient 3 showed a discrete symmetric involvement of deltoids and deep muscles of the pelvic girdle, thigh and leg. In patient 4 a CT scan performed at 34 years old

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Table 1. Summary of clinical and muscle biopsy data

Patient gender, (current age). Origin	*Onset symptoms*	*Evolution and current symptom. Walton scores*	*Biopsied muscle and age at biopsy*	*Myofibrillar/ sarcomeric disorganization*	*Purple dusty fibres*	*% NC*	*% NI*	*% NC + % NI (% MI)*	*Mean fibre diameter (SD) [Range] in µm*	*% fibres Type I*
1. Female, (15 years). Zaire	Neonatal hypotonia and respiratory distress	DMM, autonomous gait never achieved. Dysmorphism. Ophthalmopareis and ptosis. Facial diplegia, open mouth. Head support. Axial and proximal weakness. OTR absent. Hip, knee and ankle contractures. CPK: normal at birth; 241 U/l; EMG: myogenic. WS: 8. Severe.	Deltoid (4 months)	0/+	+	8.7	9.9	18.7 (3.6)	20.1 (±10.3) [4.7–43.6]	60.8
			Paravert (12 years)	+++	+++	1.5	44.9	46.3 (21)	39.7 (±53.6) [8.5–15.3]	100
			Quadriceps (14 years)	+++	+++	4.4	47.0	51.2 (22)	37.8 (±12.3) [10–64.2]	100
2. Female, (24 years). Turkey/France (Figure 2**e**–**h**)	Reduced foetal movements. Global neonatal hypotonia	DMM. Dysmorphism. Global weakness, Sporadic wheelchair bound. Ptosis, upper sight limitation. Facial paresis. Neck, masticators, elbow and ankle contractures. RRS. CPK: 204 U/l. WS: 7. Severe.	Deltoid (21 months)	+	+	2.5	1.0	3.4 (0.5)	25.5 (±7.7) [8.9–60.5]	71.4
			Deltoid (12 years)	+++	+++	7.2	23.7	30.9 (9.6)	33.8 (±13.4) [11.6–76.6]	96.1
3. Female, (24 years). France	Suction, swallowing and feeding difficulties at birth	DMM. Independent gait at 2 years. Global weakness. Eyelids weakness. Nasal voice. Masseters contracture. Currently stable, normal daily life activities. CPK: normal; EMG: myogenic. WS: 2. Mild.	Deltoid (16 years)	+	+	3.3	5.5	8.8 (0.0)	38.8 (±11.5) [18.4–75]	89.0
4. Female, (39 years). France (Figure 2**a**–**d**)	Neonatal hypotonia, poor head control	DMM. Independent gait at 18 months. Global weakness. Ophthalmoparesis. Retrognatism, talipes varus, thoracic and dorso-lumbar scoliosis. RRS. OTR absent. CPK: 26 U/l. WS: 3. Moderate.	Deltoid (37 years)	+++	+++	6.7	29.0	35.7 (10.4)	30.2 (±9.4) [10.7–63.8]	100
5. Male, (28 years). France (Figure 2**i**–**l**)	Perinatal/neonatal distress with respiratory insufficiency	DMM. Independent gait at 18 months. Dysmorphism. Ptosis, ophthalmoparesis and strabismus. Global muscle weakness. Bilateral Achillean retraction surgery. WS : 6. Severe	Deltoid (21 years)	+++	++	4.8	29.5	34.3 (21.4)	101.8 (±31.5) [31.3–194.9]	81.6
6. Male (32 years). Congo	Neonatal hypotonia	DMM. Independent gait at 2 years. Frequent falls, troubles fro climbing stairs and running. Dysmorphism, ophthalmoparesis, incomplete eyelid closing. Global weakness. CPK: 23 U/l. WS: 4. Moderate.	Deltoid (26 years)	++	+	3.3	9.0	12.3 (2.4)	36.8 (±13.4) [13.3–76]	100
7. Female (6 years). Argentina/Bolivia	Hypotonia. Suction and swallowing difficulties at birth	DMM. Independent gait at 15 months. Frequent falls. Facial hypomimia, mild bilateral ptosis. Axial and limbs weakness. Distal hyperlaxity. CPK: 64 U/l; EMG: myogenic. WS: 3. Moderate.	Deltoid (5 years)	+	0	1.3	1.3	2.7 (0.0)	29.1 (±13.4) [7.7–70.7]	95.7

Note the increase in all pathological features through sequential biopsies in patients 1 and 2.
% NI, percentage of fibres with nuclear internalization; % NC, percentage of fibres with nuclear centralization; % MI, percentage of fibres with multiple internalized nuclei; DMM, delayed motor milestones; RRS, restrictive respiratory syndrome; OTR, osteotendinous reflexes; CPK, creatine phosphor kinase; EMG, electromyography; WS, Walton score.

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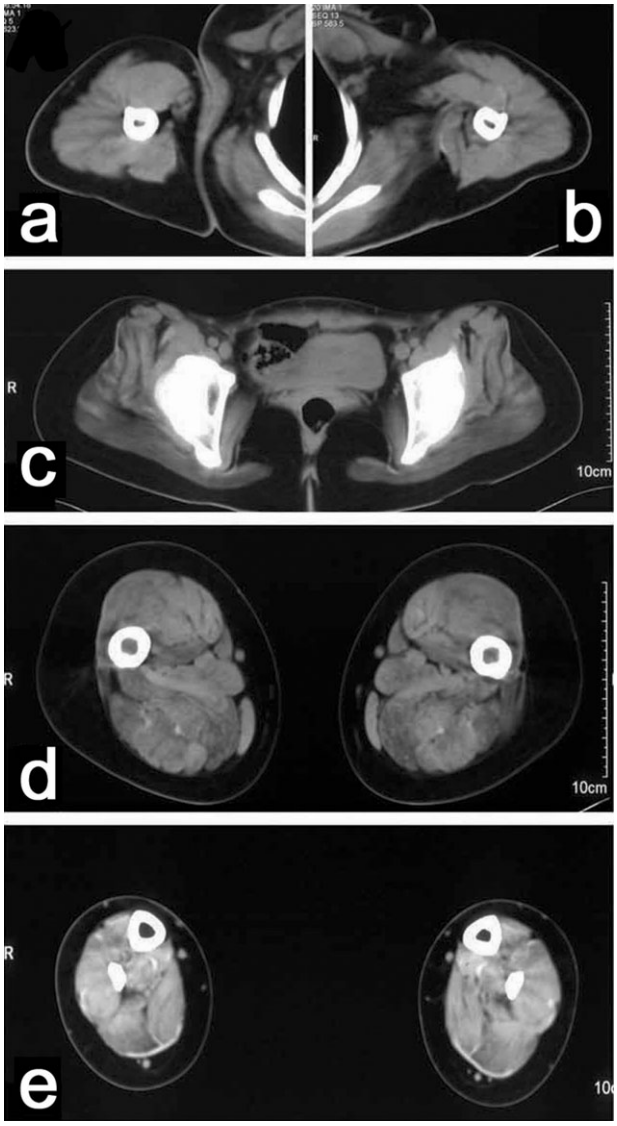

Figure 1. Computed tomography limb imaging of patient 4. Upper and lower limb computed tomography scanner from patient 4 (**a** to **e**) at the age of 34. Axial sections were performed at middle level of the arms (**a**, **b**), pelvic girdle (**c**), upper tight (**d**) and legs (**e**). In the arms and pelvic girdle, diffuse atrophy of the muscles is observed without any particular pattern. In the tights (**d**), fatty infiltration is seen in adductor magnus, semimembranosus, semitendinosus and biceps femoris, with a relative sparing of gracilis, sartorious and anterolateral compartment muscles. In the legs (**e**), hypodensity of the imaging is prominent in the internal gastrocnemius of the posterior compartment, with a relative normal signal in the other muscle.

showed a diffuse hypodensity, mainly in the tight and hamstring muscles (Figure 1). Respiratory function was severely affected in patients 1 and 2 early in life but improved slightly; their vital capacities in adolescence or adulthood were, respectively, 35% and 28% of the theoretical value (restrictive respiratory syndrome), requiring non-invasive respiratory support. Vital capacities in patients 4 and 6 were 50% and 65% of the theoretical value. Cardiac assessment was normal in all patients.

Morphological studies

Histoenzymological analyses have demonstrated a conspicuous and reliable morphological pattern on transverse muscle cryostat sections consisting of: (i) Large and weakly defined areas devoid of ATPase and oxidative activities observed in some fibres, sometimes covering the majority of the fibre diameter (Figures 2**b**,**f**,**j** and 3**g**). Such areas were identified as regions of myofibrillar and sarcomeric disorganization, either showing an absence or increased oxidative reactivity (Figures 2**c**,**g**,**k** and 3**f**). (ii) Several fibres displayed a peculiar 'purple dusty' appearance with Gomori trichrome staining, due to a precipitate of numerous small fuchsinophilic particles spreading partially or completely through the fibre cross section (Figures 2**d**,**h**,**l** and 3**d**,**h**). These extensive areas of fuzzy reddish aggregates were also evident on haematoxylin and eosin stained serial sections (Figures 2**a**,**e**,**i** and 3**e**), corresponding with the zones of abnormal staining on oxidative reactions (Figures 2**c**,**g**,**k** and 3**f**) and they also lacked ATPase activity (Figures 2**b**,**f**,**j** and 3**g**). (iii) Type I predominance or type I fibre uniformity and increased variability in fibre size; and (iv) Nuclear internalization and centralization in both fibre types, including frequent multiple internalized nuclei. In addition, a discrete increase of endomysial connective tissue was often observed.

Noticeably, the muscle biopsies performed at the ages of 4 months for patient 1 and 21 months for patient 2, essentially showed type I fibre predominance, increased endomysial connective tissue, significant variation in type I or II fibre size and the presence of some small fibres with central nuclei resembling myotubes. No cores were observed. Thereafter, the muscle biopsies performed at the ages of 12 and 14 years for patient 1 and 12 years for patient 2 showed the peculiar morphological pattern observed in all patients. Nuclear internalization increased with age (Table 1; Figure 3).

Ultrastructural findings

In patients 1 and 3 to 7, ultrastructural analysis of muscle biopsies in longitudinal sections demonstrated large areas

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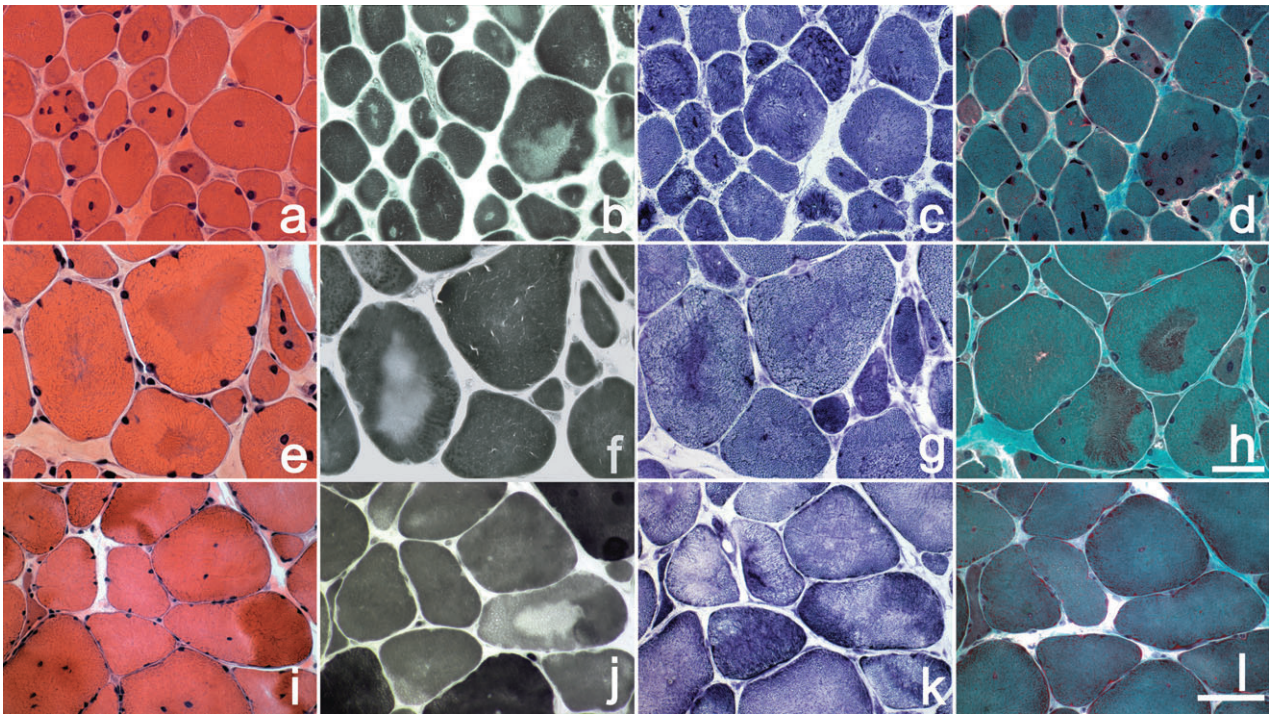

Figure 2. Light microscopy of muscle sections. Transverse serial sections of muscle biopsies from patient 4 (**a** to **d**), Patient 2 (**e** to **h**) and patient 5 (**i** to **l**) stained with haematoxylin and eosin (**a**, **e, i**), ATPase 9.4 (**b**, **f**, **j**), nicotinamide adenosine dinucleotide–tetrazolium reductase (NADH-TR) (**c, g**, **k**) and Gomori trichrome (**d**, **h**, **l**). Note with haematoxylin and eosin staining the great variability in fibre size; misplaced nuclei and multiple internalized nuclei are observed in many fibres along with increase in endomysial tissue. NADH-TR staining shows unevenness in the sarcoplasmic and mitochondrial distribution. Type I fibre predominance or uniformity is observed in all cases and some fibres show poorly delimited areas devoid of ATPase 9.4 reaction, which correspond to areas presenting sarcomeric disorganization with increased or absence of oxidative reaction. The same fibres contain the purple dusty areas with Gomori trichrome staining described in the text. Note that the two top rows show pictures at × 400 magnification, while the bottom row pictures are at × 200 magnification. (See Table 1 for details on the diameter of fibres in each case.) Scale bars: in **h** = 50 μm, for **a** to **h**; and in **l** = 100 μm, for **i** to **l**.

of sarcomeric disorganization (Figure 4**d**). Such areas were present in one or more regions within a fibre, were variable in width and length, frequently covered the entire fibre diameter in cross section (Figures 4**a**,**b**) and extended from 2 to 30 sarcomeres in longitudinal sections (Figures 4**b**,**f**). Altered fibres often showed one or several misplaced nuclei that were occasionally found at the border of areas of myofibrillar disorganization (Figures 4**b**,**d**). Within such disorganized areas, accumulation of Z-band proteins, Z-band streaming, enlarged Z-bands and myofibrillar compaction were the most frequent alterations (Figures 4**c**,**e**). T-triads-repetitions, honeycomb profiles (corresponding to T-tubules proliferations) and occasional minicore-like lesions (Figure 4**f**) were also observed amongst other non-specific alterations. Mitochondria were present or not in the disorganized areas.

Immunohistochemical findings

In order to further study the composition of the disorganized intracellular areas, biopsies of patients 2, 3 and 5 were labelled with antibodies to the intermediate filament proteins desmin, αB-crystalline and myotilin. The three markers intensively labelled the disorganized areas, but in serial sections reacting fibres were either labelled with one, two or three of the antibodies used, suggesting a heterogeneous composition of the disorganized zones (Figure 5).

RYR1 screening

Patient 1 and her deceased sister were c.[10348-6C>G; 14524G>A] + c.[8342_8343delTA] compound heterozygous carriers (Table 2). The c.8342_8343delTA frameshift deletion transmitted by the clinically unaffected

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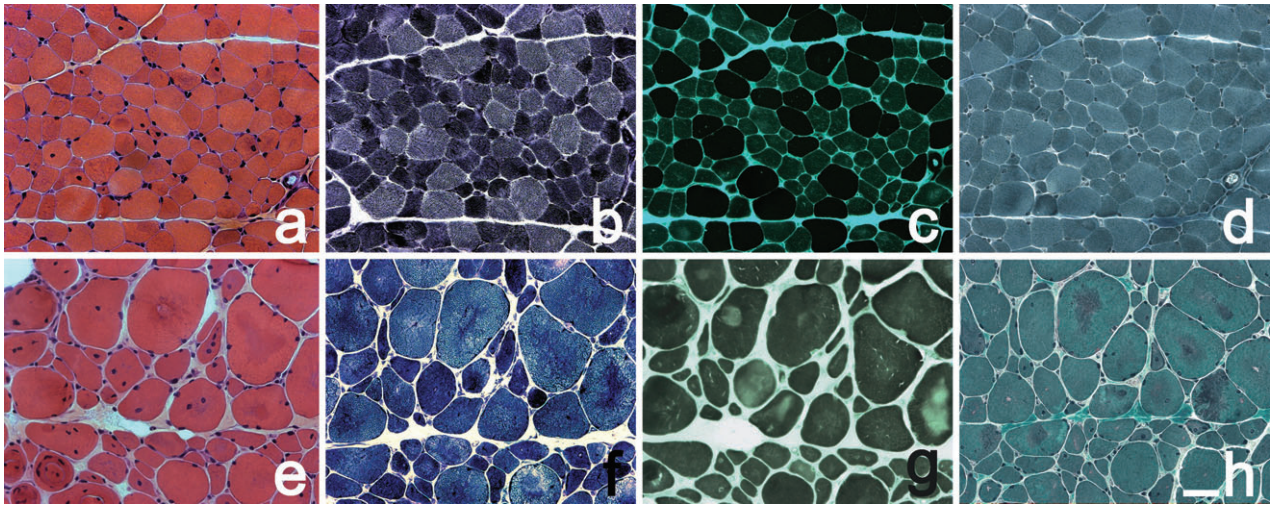

Figure 3. Evolution of histopathological findings in patient 2. Transverse serial sections of muscle biopsies from patient 2 at 21 months (**a** to **d**), and at 12 years of age (**e** to **h**) stained with haematoxylin and eosin (**a**, **e**), nicotinamide adenosine dinucleotide–tetrazolium reductase (NADH-TR) (**b**, **f**), ATPase 9.4 (**c**, **g**) and Gomori trichrome (**d**, **h**). Note with haematoxylin and eosin increase in fibre size, number of internalized nuclei and hyaline cytoplasmic protein aggregates. With NADH-TR staining the unevenness in the sarcoplasmic and mitochondrial distribution is recognized in the biopsy at 12 years but was not evident in the firs biopsy. With ATPase 9.4 the type I fibre predominance seen in the first biopsy changes to a type I uniformity and the number of fibres with areas devoid of ATPase 9.4 reaction increase in number and the areas are more conspicuous. Similar increase in the features described is seen with Gomori trichrome. (See also Table 1 for details on the diameter of fibres in each case.) Scale bar = 50 μm.

mother introduced a premature stop codon (p.Ile2781ArgfsX49). The two other variants were inherited from the clinically unaffected father. The c.10348-6C>G change resulted in a loss of splicing of intron 68 and the introduction of a premature stop codon (p.His3449ins33fsX54). Both unspliced and spliced transcripts were present, thus indicating an incomplete penetrance of this intronic variation. The c.14524G>A change in exon 101 resulted in a p.Val4842Met substitution that mapped to the M8 *trans*-membrane fragment of the Ca^{2+} pore domain [27]. RyR1 expression analysis did not show truncated proteins but instead a major decrease of the mature protein, indicating the residual presence of a low amount (15 ± 8%) of mutated Met4842 protein in the proband's muscle (Figure 6).

Patient 2 was p.[Thr4709Met] + p.[Glu4181Lys] compound heterozygous. The paternal p.Thr4709Met substitution, resulting from a c.14126C>T change in exon 96 that affected a conserved threonyl residue located in the Ca^{2+} pore domain of the protein, has been previously reported in a case of recessive core myopathy [28]. The maternal p.Glu4181Lys novel substitution that resulted from a c.12541G>A transition in exon 90, affected a highly conserved glutamyl residue located in a cytoplasmic domain of unknown function (Table 2).

Patient 3 was compound heterozygous for the novel p.[Glu4911Lys] and p.[Arg2336Cys] variants. The paternal p.Glu4911Lys (c.14731G>A, exon 102) variant affected a highly conserved glutamyl residue that mapped to the M10 *trans*-membrane fragment of the Ca^{2+} pore domain [27]. The maternal p.Arg2336Cys (c.7006C>T, exon 43) variant also substituted a very well-conserved arginyl residue located in the MH2 domain of the protein, usually associated with malignant hyperthermia dominant mutations. However, no anaesthetic history has been reported in the patient or relatives harbouring the p.Arg2336Cys variant (Table 2).

Patient 4 was p.[Pro3202Leu] + p.[Gly3521Cys] compound heterozygous. Both variants are novel and substituted highly conserved residues among species and RYR isoforms. The paternal p.Pro3202Leu (c.9605C>T, exon 65) variant affected a prolyl residue located in a central region of the protein of unknown function. The maternal p.Gly3521Cys (c.10561G>T) variant substituted a glycyl residue located within exon 71 adjacent to the alternatively spliced region I (ASI), possibly involved in interdomain interaction (Table 2) [29].

Patient 5 was p.[Pro3138Leu] + p.[Arg3772Trp] compound heterozygous. The paternal p.Pro3138Leu (c.9413C>T) variant affected a highly conserved prolyl

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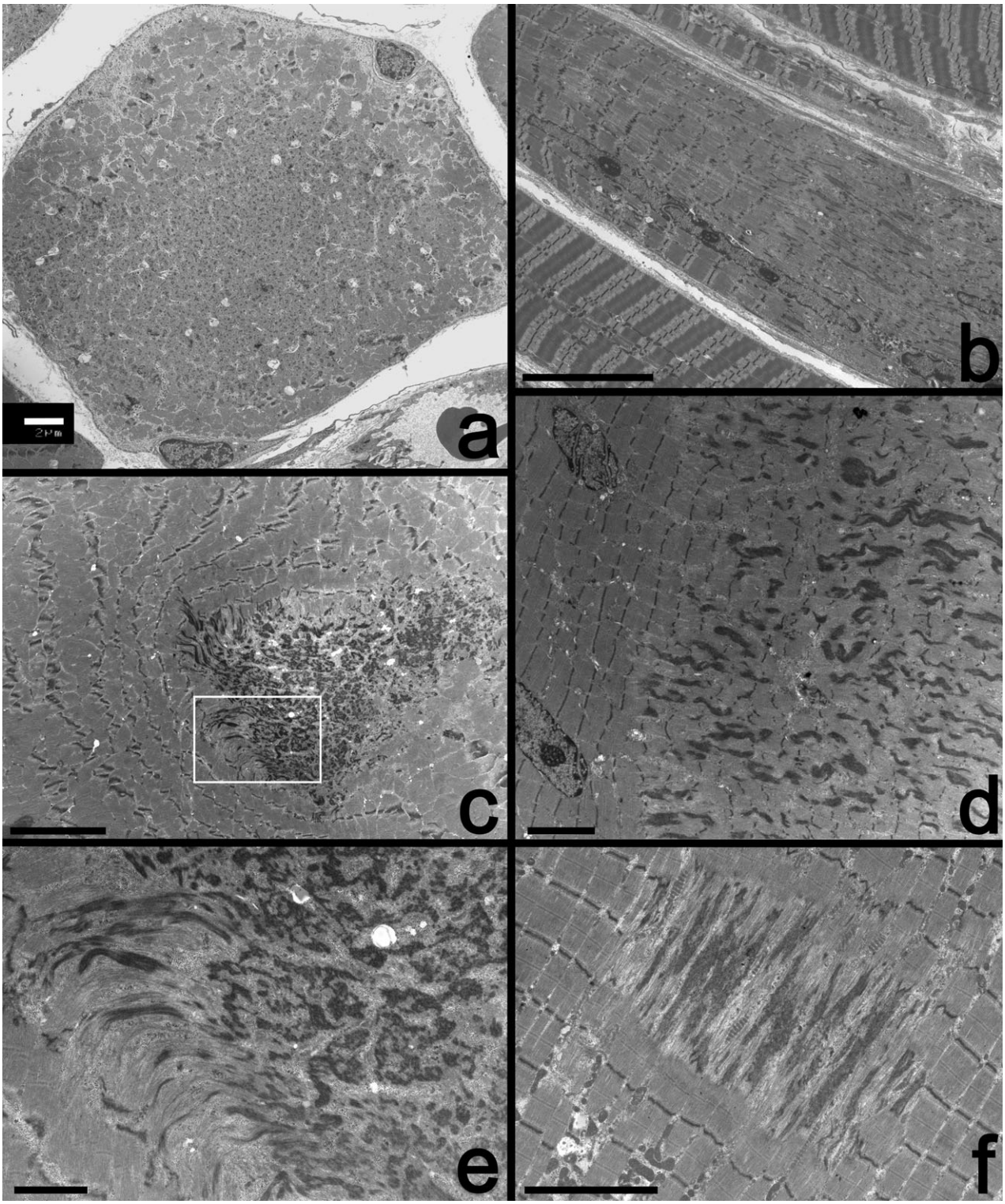

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Figure 4. Ultrastructural findings in muscle fibres from patients. Electron microphotographs of myofibres from patient 4 (**a**), patient 6 (**b**), patient 5 (**c**, **d**, **e**) and patient 3 (**f**) show different ultrastructural alterations. Transverse and longitudinal muscle sections are shown in left and right panels, respectively. The lesions cover most of the cross section of affected fibres and are adjacent to internalized nuclei (**a**, **b**). The areas of myofibrillar disorganization contain Z band proteins aggregates (**c**, **e**). The box framed in **c** is depicted at higher magnification in **e**. Two misplaced nuclei are near the border of an area of myofibrillar disorganization, which continues from normally appearing sarcomeres (**d**). Triad repetitions are seen within a small zone of Z disc streaming (**f**). Scale bars in **a** and **e** = 2 μm, **b** and **c** = 10 μm, **d** = 4 μm and **f** = 5 μm.

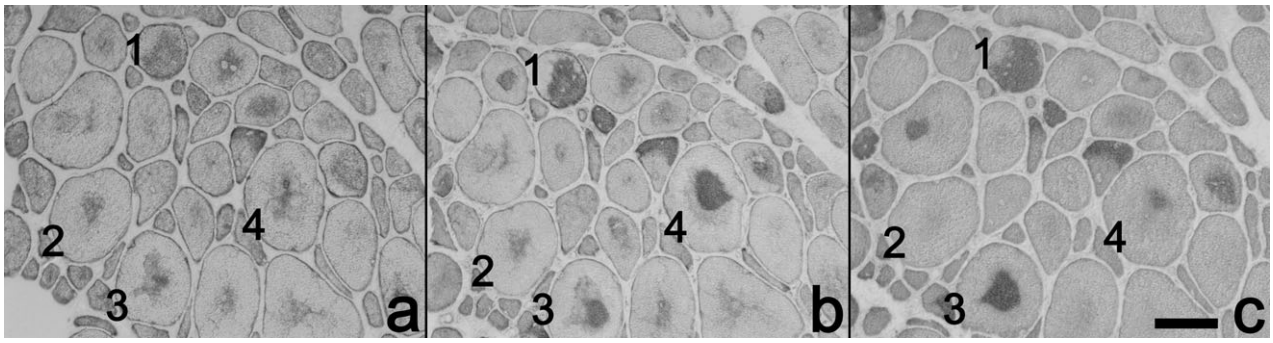

Figure 5. Muscle biopsy immunostaining. Serial sections immunostained with antibodies to desmin (**a**), αB-crystalline (**b**) and myotilin (**c**) showed positive staining for all the markers used. In some cells labelling was similar with the three markers (1); in other, reaction was positive for desmin and αB-crystalline but not myotilin (2), and in a third set of fibres labelling was somehow stronger for myotilin (3), whilst in a fourth group of fibres labelling was stronger for αB-crystalline (4). Scale bar in **c** = 30 μm.

residue that mapped to exon 63. This variant has not been reported previously. The maternal p.Arg3772Trp (c.11314C>T, exon 79) variant has been recently reported in an MHS patient [30]. The mutation substituted a highly conserved argininyl residue into a nonconservative tryptophan located in a cytoplasmic domain of unknown function (Table 2).

Analysis of patient 6's cDNA revealed the presence of two abnormal transcripts characterized by insertions of 132 bp and 32 bp between exons 56 and 57, and the presence of a normally spliced transcript. Genomic sequencing of intron 56 identified a homozygous c.8692+131G>A change, which revealed a cryptic donor splice site in competition with the physiological splice site (Table 2). Use of this cryptic splice site led mostly to an insertion of 132 bp that introduced 44 amino acids and a premature stop codon between exons 56 and 57 (p.Gly2898GlyfsX36). In addition, the presence of another putative AG dinucleotide splice acceptor site upstream to the cryptic donor splice site, led to an additional alternative frameshift insertion of 32 nucleotides, also leading to a premature stop codon (p.Gly2898AspfsX54) (Figure 7**a**). However, no truncated proteins were detected on Western blot analysis, suggesting either instability of the cryptic transcripts as a result of a nonsense-mediated mRNA decay process or an early degradation of the truncated proteins as a result of an unfolded protein response. The residual physiological splicing allowed the production of a low amount of wild-type RyR1 (22 ± 12%) in the muscle of the patient (Figure 6).

Patient 7 was p.[Pro3202Leu] + p.[Arg4179His] compound heterozygous. The maternal p.Pro3202Leu (c.9605C>T, exon 65) variant was recurrent in this study (patient 4). The paternal p.Arg4179His (c.12536G>A, exon 90) variant affected a highly conserved arginyl residue that mapped to a cytoplasmic domain of the protein close to the p.Glu4181Lys variant identified in patient 2.

Discussion

We have identified a cohort of seven patients with congenital myopathy and a peculiar morphological pattern in muscle biopsies associated with recessive mutations in the gene encoding the skeletal muscle ryanodine receptor (*RYR1*). All the patients showed early onset of the disease, ophthalmoparesis of variable severity and presence of early disabling contractures, especially in the masticators. Rigid spine syndrome was also present in two patients. Otherwise clinical presentation was similar to most congenital myopathies, showing hypotonia of variable severity, delay in the acquisition of developmental motor

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Table 2. Summary of molecular genetic findings

Patient	*Localization*	*Nucleotide changes (cDNA numbering)**	*Origin of alleles*	*Amino acid changes*	*Expected consequences*	*References*
1	Intron 68	c.10348-6C>G	Father	p.His3449ins33fsX54	Truncated protein	Monnier *et al.* 2008 [19]
	Exon 101	c.14524G>A	Father	p.Val4842Met	Missense mutation	Monnier *et al.* 2008 [19]
	Exon 53	c.8342_8343delTA	Mother	p.Ile2781ArgfsX49	Truncated protein	This study
2	Exon 96	c.14126C>T	Father	p.Thr4709Met	Missense mutation	Zhou *et al.* 2006 [28]
	Exon 90	c.12541G>A	Mother	p.Glu4181Lys	Missense mutation	This study
3	Exon 102	c.14731G>A	Father	p.Glu4911Lys	Missense mutation	This study
	Exon 43	c.7006C>T	Mother	p.Arg2336Cys	Missense mutation	This study
4	Exon 65	c.9605C>T	Father	p.Pro3202Leu	Missense mutation	This study
	Exon 71	c.10561G>T	Mother	p.Gly3521Cys	Missense mutation	This study
5	Exon 63	c.9413C>T	Father	p.Pro3138Leu	Missense mutation	This study
	Exon 79	c.11314C>T	Mother	p.Arg3772Trp	Missense mutation	Levano *et al.* 2009 [30]
6	Intron 56	c.8692+131G>A	Father and mother	p.Gly2898GlyfsX36 + p.Gly2898AspfsX54	Truncated proteins	This study
7	Exon 65	c.9605C>T	Mother	p.Pro3202Leu	Missense mutation	This study
	Exon 90	c.12536G>A	Father	p.Arg4179His	Missense mutation	This study

The mutation, origin of mutated *RYR1* allele and expected change at the protein level are indicated for each patient. See also Figures 4 and 5.
*GenBank NM 000540.2 with +1 corresponding to the A of the ATG translation initiation codon.

Figure 6. Western blots analysis of muscle biopsy. Forty micrograms (40 µg) of muscle homogenate from control (C), patient 1 (P1) or patient 6 (P6) have been loaded on a 5–15% acrylamide gel, and after electrotransfert to Immobilon-P, the blot has been incubated with an anti-RyR antibody (Marty *et al.* [26]) which cross react with the full length protein (the higher molecular weight band in the control) and the degradation fragments (the lower molecular weight bands in the control). Only the full length protein is detected in both patient biopsy, which represent 22% ± 12% (P6) and 15% ± 8% (P1), respectively, of the control.

milestones, axial and proximal limb weakness and restrictive respiratory syndrome. Cardiac and cognitive functions were invariably spared.

Our data enlarges the histological phenotype associated with *RYR1* mutations. Indeed, the areas of sarcomeric/myofibrillar disorganization are distinguishable from typical cores. On oxidative stains, these areas are large, diffuse and poorly delimited. Ultrastructurally, they are broader than cores in transverse sections, as they frequently cover extensive cross-sectional areas of the fibre, often reaching the sarcolemma. They are also shorter than cores, as in longitudinal sections they extend along a relatively small number of sarcomeres. In contrast with cores the presence of mitochondria within the lesions accounts for the excessive oxidative staining in some fibres. On the other hand, 'purple dusty areas' corresponding to foci of Z line rearrangements are not usually seen in muscle biopsies of patients with classical core myopathies. Interestingly, the first descriptions of congenital myopathies with 'target-like fibres' as described by Schotland in the 1960s, reported morphological findings close to those described in our patients; retrospectively, these similarities might provide the molecular cause for these earlier observations [31,32].

Moreover, in our series of patients, nuclear misplacement affected up to 51% of the fibres. Remarkably, fibres with centralized nuclei ranged from 1 to 9%, while nuclear internalizations were present in up to 47% of the fibre population, of which up to 22% had multiple

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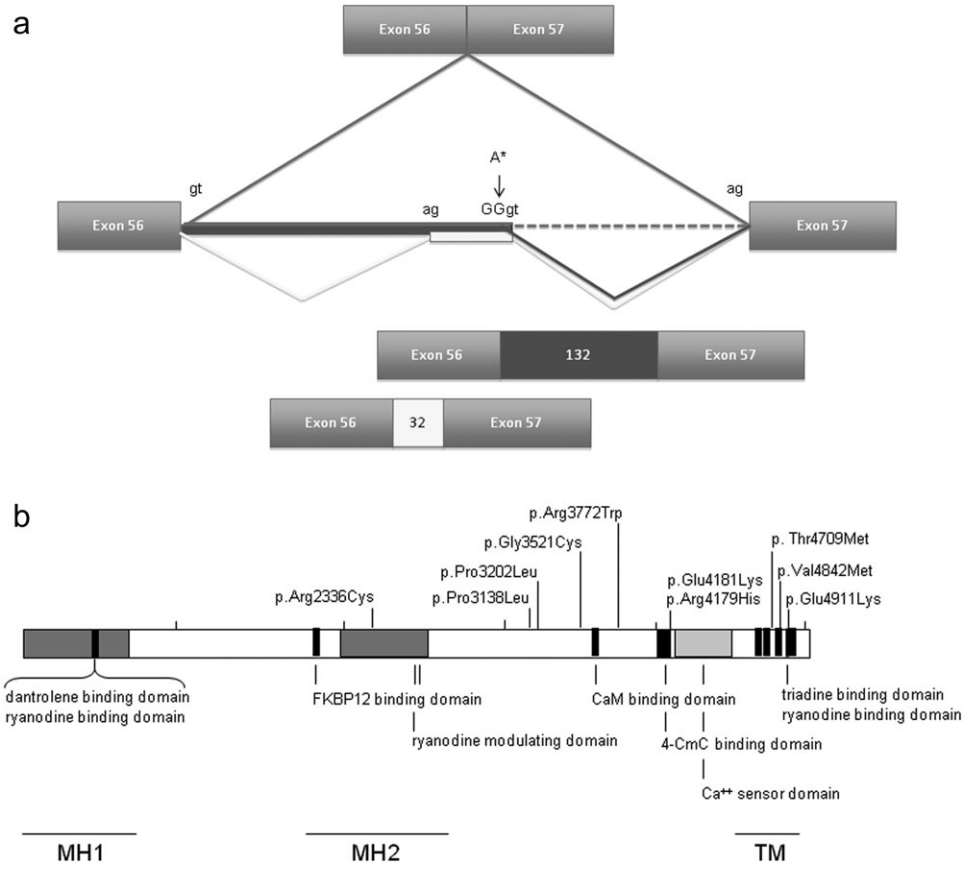

Figure 7. Schema relating to mutations found in patient 6 and summary of *RYR1* mutations. (**a**) Consequences of the homozygous c.8692+131G>A mutation in *RYR1* intron 56 identified in patient 6. (**b**) Mapping of the missense mutation in *RYR1*. MH1 and MH2, hot spots for mutations associated with malignant hyperthermia (MH) susceptibility; TM, transmembrane domains; CaM, calmodulin; 4-CmC, 4-chlorocresol. Seven missense variants were novel (p.Arg2336Cys, p.Pro3138Leu, p.Pro3202Leu, p.Gly3521Cys, p.Arg4179His, p.Glu4181Lys, p.Glu4911Lys); the p.Pro3202Leu variant found in two unrelated patients in this study (patients 4 and 7).

internalized nuclei (Table 1). This contrasts with what is usually observed in *DNM2*-, *BIN1*- and neonatal *MTM1*-related CNM, where fibres with centralized nuclei clearly outnumber fibres with internalized nuclei [24]. In addition, in this set of recessive *RYR1*-related patients, internalized nuclei are frequently multiple, and are randomly dispersed into the sarcoplasm. As we have stressed in previous reports [24,25,33] and confirmed in the present study, the location of misplaced nuclei (that is, central, random, unique, multiple) is a relevant clue to orientate molecular diagnosis.

Interestingly, a pathophysiological link has been suggested between *RYR1* and CNM based on the study of a *MTM1* knock out mice, which presented reduced levels of RyR1 protein and defects in excitation–contraction coupling [34]. We assessed MTM1 protein content in muscles from our recessive *RYR1*-related patients but no variation was found with respect to control samples (data not shown).

As the areas of myofibrillar disorganization described here in some muscle fibres appear to lack ATPase and oxidative activities, such structural rearrangements could be mistakenly interpreted as similar to the 'rubbed-out fibres' usually observed in myofibrillar myopathies, therefore suggesting a pathological overlap between the two myopathies. However, the structural alterations are different especially at the ultrastructural level [24,35]. In addition, the clinical, muscle imaging and pathological context of patients should be considered in the differential diagnosis.

The notion that histoarchitectural changes in congenital myopathies evolve according to age is not novel.

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Several reports have addressed the topic, both before and during the molecular genetics era [9,17,20,36,37]. However, the marked alterations described in the biopsies of patients 1 and 2 of this series deserve a special consideration, as they may lead to an inappropriate diagnosis. Thereby, after the first years of life, the pattern of alterations evolved towards those of a congenital myopathy (that is, type I predominance and hypotrophy, type I uniformity, low percentage of internalized nuclei), to finally consolidate during the second half of the first decade, into the typical pattern of alterations described herein (core-like lesions, purple dusty fibres, multiple internalized nuclei) (Figure 3). Such considerations are of great relevance for the pathological differential diagnosis. However, the histological hallmark in these cases was the presence of the unclearly delimited areas of myofibrillar disorganization observed with oxidative and ATPase techniques. These alterations, which were less conspicuous and affected fewer fibres in younger patients, were nonetheless the right clue to direct molecular testing.

Our data significantly enlarges also the spectrum of *RYR1* mutations since; among the 13 variants identified, nine are novel (Table 2 and Figure 7**b**). Compound heterozygous mutations were identified in six unrelated patients and a homozygous mutation in patient 6. Compound missense mutations were present in five patients while amorphic/hypomorphic mutations leading to RyR1 depletion were found in two patients (patients 1 and 5). In six patients recessive inheritance was confirmed by familial studies. In patient 6 for whom parental samples were not available, familial consanguinity, homozygosity of the mutation and the absence of familial history were strongly suggestive of a recessive inheritance.

Seven missense variants were novel. All of them were absent in 200 unrelated controls and affected highly conserved residues. The p.Thr4709Met variant has been already reported in a recessive form of core myopathy [28] while the p.Arg3772Trp change has been identified as the single change in *RYR1* in an MHS patient [30]. This last variant, which is clearly recessive with respect to the myopathy, could confer dominant MHS susceptibility. This could be also the case of the p.Arg2336Cys variant that mapped to the MH2 domain of the protein, a hot spot for malignant hyperthermia mutations, and whose position has already been involved in a malignant hyperthermia-causing mutation (Arg2336His) [30]. Most of the variants present in this study were located in the cytoplasmic region spanning from the MH2 domain to the Ca^{2+} pore domain whose functions remain mostly unknown. Moreover, the pathophysiological pathways associated with recessive missense mutations in *RYR1* are generally unknown and are likely to be mutation specific [38]. No malignant hyperthermia reactions were documented in these patients or among their relatives; however, *in vitro* contracture testing was not carried out in this series. Nevertheless, awareness about the potential risk of MHS is advisable before affected patients or their possible carrier relatives.

Patient 1 was compound heterozygous for a null mutation (c.8342_8343delTA) on one allele and for a hypomorphic splicing mutation (c.10348-6C>G) associated with a missense variant (p.Val4842Met) on the second allele. Only a low amount of Met4842 mutant RyR1 protein was detected in muscle biopsy. Interestingly, a low amount of Met4842-RyR1 protein has previously been observed in two affected sisters who were compound heterozygous for the same missense and other null mutations [c.10348-6C>G, p.Val4842Met] and a c.7324-1G>T [19]. They also presented a severe neonatal form of congenital myopathy. In contrast, patient 6 was homozygous for the hypomorphic c.8692+131G>A mutation. The severity of his disease was relatively moderate despite the low amount of RyR1 expressed in the muscle, but it should be noted however that the residual expression corresponded to the wild-type RyR1 protein in this patient. Altogether these data suggest that RyR1 depletion in skeletal muscle is one of the pathophysiological mechanisms of the disease as already reported in recessive forms of *RYR1*-related congenital myopathy [19,28,38–40].

In conclusion, we have identified a specific clinical and histological phenotype associated with recessive *RYR1* mutations. Our data clearly show that in this group of patients, the histological phenotype shares features traditionally described in different forms of congenital myopathies, namely centronuclear and core myopathies. They strongly support the idea that the presence of disorganized myofibrillar areas with irregular borders in muscle biopsies from patients with clinical manifestations of congenital myopathy are likely to be due to *RYR1* mutations, even in the presence of numerous fibres with internalized nuclei. Hence, this peculiar morphological pattern should be consistently associated with the subgroup of 'congenital myopathies with cores'. This will improve molecular diagnosis and consequently, genetic counselling and the prognosis given to patients.

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