Caveolinopathy - new mutations and additional symptoms

Ahmed Aboumousa¹,⁷; Jessica Hoogendijk¹,⁶; Richard Charlton¹; Rita Barresi¹; Ralf Herrmann²; Thomas Voit³; Judith Hudson¹; Mark Roberts⁴; David Hilton-Jones⁵; Michelle Eagle¹; Kate Bushby¹; Volker Straub¹

Affiliations
¹Institute of Human Genetics, University of Newcastle upon Tyne, UK
²Department of Paediatrics and Paediatric Neurology, University Hospital Essen, Germany
³Institut de Myologie, Groupe Hospitalier Pitié-Salpêtrière, Université Pierre et Marie Curie Paris VI, France
⁴Department of Neurology, Withington Hospital, Manchester, UK
⁵Department of Clinical Neurology, West Wing, John Radcliffe Hospital, Oxford, UK
⁶Department of Neurology, Rudolf Magnus Institute of Neurosciences, University Medical Centre Utrecht, the Netherlands
⁷Department of Neurology, Kasr Al-Aini Faculty of Medicine, Cairo University, Egypt.

Address for correspondence:
Professor Volker Straub
University of Newcastle upon Tyne
Institute of Human Genetics
International Centre for Life
Central Parkway
Newcastle upon Tyne, NE1 3BZ, UK
Tel.: +44 (0)191 241 8762/8655
Fax: +44 (0)191 241 8770
email: volker.straub@ncl.ac.uk

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Abstract

Mutations in the caveolin-3 gene (CAV3) can lead to a broad spectrum of clinical phenotypes. Phenotypes that have so far been associated with primary caveolin-3-deficiency include limb girdle muscular dystrophy, rippling muscle disease, distal myopathy and hyperCKemia. This is the first report describing the clinical, pathological and genetic features of patients with caveolinopathy from the UK. Ten patients (six families) were identified via the National Commissioning Group (NCG) service for patients with limb girdle muscle dystrophy in Newcastle. Myalgia was the most prominent symptom in our cohort of patients and for 50% it was the reason for referral. Muscle weakness was only found in 60% of the patients, whereas rippling muscle movement was present in 80%. One of the patients reported episodes of myoglobinuria and another one episodes of hypoglycaemia. Five different mutations were identified, two of which were novel and three that had previously been described. Caveolinopathy needs to be considered as a differential diagnosis in a range of clinical situations, including in patients who do not have any weakness. Indeed, rippling muscles are a more frequent symptom than weakness, and can be detected in childhood. Presentation with myalgia is common and management of it as well as of myoglobinuria and hypoglycaemia may have a major impact on the patients’ quality of life.
Introduction

Caveolae ('little caves'), are 50 to 100 nm small membrane invaginations on the surface of cells, which represent appendages or subcompartments of plasma membranes [1]. They participate in membrane trafficking, sorting, transport and signal transduction, including endocytosis and potocytosis. Caveolin is believed to play a role in the formation of the caveolae membranes, acting as a scaffolding protein that organizes and concentrates caveolin-interacting proteins and lipids in caveolae microdomains [2]. The mammalian caveolin gene family consists of three caveolins, caveolin-1, -2 and -3. Caveolins-1 and -2 are co expressed in adipocytes, whereas the expression of caveolin-3 is muscle specific [3].

Caveolin-3 is a 21-24 kDa integral membrane protein formed of 151 amino acids (aa). Three separate segments can be identified in this protein, an N-terminal region (aa 1-73), a central hydrophobic transmembrane domain (aa 75-106) and a C-terminal (aa 107-151) domain. The transmembrane domain is believed to form a hairpin loop structure in the cell membrane, allowing both the N- and C-terminal ends to face the cytoplasm. The N-terminal domain contains a caveolin signature sequence (aa 41-48, FEDVIAEP present in all caveolins) and a scaffolding domain (aa 55-74) known to bind various signalling proteins. It is also responsible for the homo-oligomerization and the interaction with caveolin-associated signalling molecules such as eNOS, β-adrenergic receptors, protein kinase C isoforms, G proteins, Src-family kinases, and multiple components of the dystrophin-glycoprotein complex [4, 5].
Mutations in the CAV3 gene have been described in different domains of the protein. They are usually inherited in an autosomal dominant pattern and are associated with a broad spectrum of clinical phenotypes including LGMD1C, distal myopathy, rippling muscle disease (RMD) and hyperCKaemia. Recently, an autosomal recessive inheritance of some CAV3 mutations was considered [6-9].

Different pathogenic mechanisms have been postulated to explain muscle pathology in caveolin-3 deficient muscles. First, it was found that mutated caveolin-3 behaved in a dominant-negative fashion, causing the retention of wild type protein at the level of the Golgi apparatus [10]. Secondly, altered caveolin-3 expression changed the outcome of phosphoinositol-3-kinase activation from cell survival to cell death [11]. Thirdly, caveolin-3 null mice showed T-tubule abnormalities that could lead to dysregulation of muscular calcium homeostasis initiating a dystrophic process and possibly RMD [3].

Here we describe the clinical details of the first group of patients with caveolinopathy from the UK representing six families with five different mutations. Our cohort of patients showed various phenotypes that extend the clinical spectrum of patients with caveolin-3 deficiency. In addition, we found two novel mutations in the caveolin gene in our patients.
Patients and methods

The Institute of Human Genetics in Newcastle Upon Tyne, UK serves as the national referral centre for patients with a possible diagnosis of limb girdle muscular dystrophy. The service is commissioned by the National Commissioning Group (NCG), covers residents from England, Wales and Scotland and provides clinical assessment, biopsy analysis and genetic testing.

We describe 10 patients (3 male and 7 female) from six families with genetically confirmed mutations in the CAV3 gene. All patients underwent a thorough neurological assessment and six of them (one patient from each family) had a muscle biopsy. The manifestations of mechanical irritability of the muscles (percussion induced rapid contractions/PIRCs, mounding and rippling muscle movements) were assessed as previously described [12]. Serum creatine kinase levels were measured in all patients. Six patients had electrophysiological investigations.

Muscle biopsy studies: Muscle biopsies were assessed by routine histochemistry and immunoanalysis. Optimised immunohistochemical and multiplex western blot protocols were used as previously described[13, 14].

Commercial antibodies to the laminin α5 chain (mAb 1924), laminin β1 chain (mAb 1921), laminin γ1 chain (mAb 1920) and Collagen VI (mab 1944) were obtained from Chemicon International (Temecula, CA). Antibodies against α-dystroglycan (clone IIH6) was a gift from Kevin Campbell, β-spectrin (RBC2/3D5), β-dystroglycan (43DAG/8D5), C-terminal dystrophin (Dy8/6C5) and the N-terminal dystrophin (DY10/12B2), α-sarcoglycan (Ad1/20A6), β-sarcoglycan (1/5B1), γ-sarcoglycan (35DAG/21B5), δ-sarcoglycan (3/12C1), neonatal Myosin (MHCn), laminin α2 chain
(NCL-Merosin) and dysferlin NCL-Hamlet /NCL-Hamlet were all from Novocastra. Caveolin-3 C38320 was supplied by Becton Dickinson.

Briefly, sections were washed for 15 minutes in PBS pH 7.3 containing 0.1% Triton X to permeabilise the membranes. Excess buffer was removed and sections incubated overnight at 4°C in optimally diluted primary antibody diluted in 40% Foetal Calf Serum containing 0.1M lysine. Following 2x10 minute washes in PBS/Triton sections were incubated for 90 minutes at room temperature in HRP rabbit anti mouse IgG (Dako P260) diluted 1:100 in 0.1M lysine in 40% Foetal Calf Serum. Sections were washed for 2x10 minutes as above, visualised with 3,3'-diaminobenzidene (DAB) and counterstained with Carazzi’s haematoxylin prior to dehydration and mounting. Control sections were labelled without primary antibodies, and all sections were compared with control samples from other neuromuscular disorders, and with normal muscle.

Multiplex Western blot analysis was performed using biphasic polyacrylamide gradient gels 4-12% gels and antibodies against dysferlin (NCL-hamlet), calpain 3 (Calp3d/2C4 and Calp3c/12A2), dystrophin (Dy8/6C5 C-terminus and Dy4/6D3 rod domain), α-sarcoglycan (Ad1/20A6), β-dystroglycan (43DAG/8D5), γ-sarcoglycan (35DAG/21B5), the laminin α2 chain (Chemicon MAB 1922) and Caveolin-3 C38320. Myosin heavy chain staining on the post blotted gel was used to indicate how much actual muscle protein (as opposed to fat and fibrous connective tissue) was loaded in each lane[13].

**Mutation analysis.**

Genomic DNA (50–100 ng) extracted from peripheral blood lymphocytes was used as a template for PCR amplification. Reactions with exon-specific primers were
performed as previously described [6]. Thirty-five cycles of amplifications were
performed as described above with an annealing temperature of 55°C[6]. PCR
products were digested with BclI (LifeTechnologies, Karlsruhe, Germany) and
products were analyzed by electrophoresis on a 2% Seakem LE agarose (FMC,
Rockland, ME) gel stained with ethidium bromide.

Bi-directional fluorescent sequencing was performed using dye terminator cycle
sequencing chemistry and analysed on either 373A Fluorescent Automated
Sequencer (Applied Biosystems) or a CEQ8000 Genetic Analysis System (Beckman
Coulter).
Results

Clinical history and examination

In our current study, we analyzed the clinical history, clinical presentation and other diagnostic findings of 10 patients with mutations in their CAV3 gene. Table 1 summarizes the clinical findings in our patients.

The mean age at onset of symptoms was 14 years (range 5 - 32 years). The first disease related complaints (presenting symptoms) included myalgia, toe walking, muscle weakness and rippling muscle movements. Muscle pain was the presenting symptom in five patients. Pain was associated with cramps (spontaneous or post exertion) and was described as a dull aching pain. Severity of pain ranged from mild muscle discomfort to almost unbearable pain. Pain in one patient (patient 8) was so severe that it was insufficiently controlled with 450 mg tramadol hydrochloride plus 4 g paracetamol per day. Other presenting symptoms included toe walking, rippling muscle movements, and large chunky calves. None of the patients presented with respiritory or cardiac symptoms.

One patient (patient 4) reported several episodes of myoglobinuria (acute muscle cramps, myalgia and pigmenturia triggered by exercise) at around 20 year of age. Interestingly this patient did not show evidence of weakness at the time of examination. To our knowledge, this is the first time myoglobinuria has been reported in a patient with caveolinopathy. Another patient (patient 3) described multiple hypoglycaemic episodes for many years. On sustained aerobic exercise, she developed symptoms of dizziness and became sweaty. These symptoms also occurred after occasional fasting periods. They were aborted by eating something containing sugar. Her blood glucose level reached 3.0 mmol during one of these episodes. She reported that her half brother (who has identical muscle symptoms)
also has similar episodes. Chronic (cyclic) neutropenia was reported in another patient. Her neutrophil count has been as low as $2.3 \times 10^9$. Despite extensive haematological investigations no definite cause for her neutropenia was identified and it remains unclear if this related to the caveolinopathy.

The duration of the disease at diagnosis varied between 3 to 41 years. All patients had noticed muscle hypertrophy especially in the calf muscles during the course of their disease. One patient presented with bilateral wasting of small hand muscles. Eight patients reported rippling movements in their muscles. Rippling was induced by applying pressure to a stretched muscle. Most patients knew how to induce rippling. Mounding was present in two of those patients. Most of the patients who showed rippling movements did also show percussion induced rapid contractions (PIRCs, [12]), which were easily elicited in both muscles of the upper and lower limbs. Proximal weakness affecting both the upper and lower limbs was found in six patients. The weakness was bilateral and symmetrical. Its degree varied from MRC grade 4 in the shoulder girdle and grade 3-4 in the pelvic girdle muscles. Two patients showed associated MRC grade 4 weakness of the interossei muscles and one of them had associated weakness of the thumb muscles. In the three patients presenting with toe walking there was no evidence of distal weakness in the lower legs. Interestingly four of our patients showed no muscle weakness at all after a disease history of 13-31 years. Two of these patients showed mild finger flexion contractures and tight hamstrings. There were no signs of facial weakness, scapular winging or spinal rigidity in our patients.
**Family history**

Family history analysis helped to identify the autosomal dominant pattern of inheritance in three families and allowed identification of other affected family members. Intrafamilial phenotypic variability was seen in two families. In one of the families, a mildly affected mother without rippling muscle disease had four affected children with rippling muscle disease. In another family the affected mother (patient 5) presented with weakness without evidence of rippling movements, whereas her affected children presented with rippling movements without evidence of weakness (patient 6) and with toe walking (patient 7). Patient four reported that his mother, sister, son and daughter all showed toe walking and calf hypertrophy, suggestive of caveolinopathy.

**Diagnostic investigations (electrophysiology, CK)**

The results of the electrophysiological assessments, CK values and muscle biopsies are presented in table 2. Serum CK levels were elevated in all patients. CK levels were elevated three to thirty fold. In eight patients, CK levels were nine fold above normal. EMG was done in six patients. All patients showed myopathic changes. Two patients showed spontaneous fibrillation potentials and positive sharp waves at rest.

**Muscle morphology and immunoanalysis:**

Index cases in each family underwent a muscle biopsy. The histopathological features of the muscle varied from dystrophic appearance (variation in fibre size, fibre splitting, internal nuclei, fibre necrosis and regeneration) to biopsies without any abnormalities. In patient 5 H&E staining showed rimmed vacuoles in addition to other
dystrophic changes. Interestingly this patient showed evidence of distal weakness and wasting. None of the biopsies was reported to show excess glycogen storage on Periodic acid-Schiff (PAS) staining. The immunoanalysis of skeletal muscle sections showed absent to weak caveolin staining (figure 1). This was associated with secondary reduction in dysferlin staining in two biopsies. Dysferlin staining was within normal range in the rest of the biopsies. Immunoblotting showed absent caveolin in three patients. Immunoblotting of the other proteins used in the panel, including dysferlin, was normal (figure 1).

**Results of CAV3 gene analysis:**

In our group of patients, we found two novel missense mutations in the CAV3 gene in two unrelated families. The sequence variants were not found in control samples. The first mutation is 141 G/T in exon 2 leading to the substitution of glutamine by aspartate at position 47 of the caveolin signature domain. This mutation was found in two sisters. The second mutation is 218 A/G in exon 2 leading to the substitution of tryptophan by cysteine at position 73 of the scaffolding domain of the protein. This mutation was found in one patient. The mutation in the first patient (301 T/C leading to the substitution of tryptophan by arginin in position 101 of the transmembrane domain of the caveolin-3 protein) has previously been reported [15]. The substitution of 80 G>A in exon 1 causing an amino acid change of arginin to glutamine in position 27 in the N-terminal domain of caveolin was reported in one family and it was described before in a patient with reduced caveolin-3 expression and rippling muscle disease [16]. The substitution of 136G>A in exon 2 causing an amino acid change of alanine to threonine in position 46 in the caveolin-signature
domain was identified in two families (patients 5, 6, 7 and patient 10). This mutation has been described before [17, 18].
Discussion

Mutations in the CAV3 gene can cause a wide spectrum of clinical phenotypes including hyperCKaemia, rippling muscle disease, distal myopathy and limb girdle muscular dystrophy (LGMD1C) [19]. Many patients show an overlap of these symptoms and it has therefore been suggested to use the term caveolinopathy for patients with a primary caveolin defect.

In our cohort of patients, muscle pain was one of the main presenting symptoms and had a bigger impact on quality of life than muscle weakness. Caveolinopathy should always be included in the differential diagnosis of myalgia as it can be the only clinical symptom of a CAV3 mutation. The presence of muscle pain in caveolinopathies may be explained by impaired glucose metabolism in muscle and a shift to anaerobic glycolysis. It has recently been reported that in the absence of caveolin-3 phosphofructokinase (PFK), a key regulatory enzyme in the glycolytic pathway, is no longer targeted to the plasma membrane and is excluded from caveolar membrane domains [20, 21]. Patients with phosphofructokinase-deficiency (Glycogenosis type VII) do also frequently experience myalgia. The mislocalisation of phosphofructokinase in caveolinopathy could lead to an impaired glucose metabolism and might explain some of the clinical symptoms associated with the disease [21].

The biopsies of our cohort of patients showed no abnormalities on PAS-staining. PFK analysis was not performed on the patients' biopsies. Interestingly one of our patients experienced episodes of myoglobinuria, which to our knowledge has not been reported before in caveolinopathy. The postulated phosphofructokinase mislocalisation may also play a role in the pathogenesis of this symptom as myoglobinuria is a common feature in patients with phosphofructokinase deficiency.
Myoglobinuria and its sequelae should therefore be discussed with patients with caveolinopathy.

Another one of our patients developed well documented episodes of hypoglycaemia and so did a family member who had identical muscle symptoms. This observation suggests that these hypoglycaemic episodes could be related to the caveolinopathy. This would also be in accordance with recent reports on caveolin-3 deficient mice, which showed that caveolin-3 null mice develop insulin resistance [22, 23]. Proper assessment of glucose homeostasis in patients will be necessary to better characterize abnormalities of glucose metabolism in caveolin-3-deficiency.

The presence and distribution of muscle weakness in our patients was variable. Forty percent of the patients showed no signs of muscle weakness as a manifestation of their disease. In the remaining patients, weakness was affecting limb girdle muscles. Two of the patients showed associated distal weakness in the interossei and thumb muscles. We found that there was no correlation between the development of weakness and the duration of the disease. Some patients developed weakness within 2 years of onset and other not even after 30 years.

Manifestations of muscle hyperirritability (Rippling muscle movements and PIRCs) were found in eight patients. Neither PIRCs nor rippling was necessarily seen as a clinical problem by the patients. Both PIRCs and rippling movements were highly suggestive of CAV3 gene abnormalities in the patients. On the other hand there are patients with rippling muscle movements without mutations in CAV3 gene [24]. With regard to recent reports on immune-mediated RMD it is important to remember that other diseases like myasthenia gravis can be associated with secondary caveolin-3
deficiency. Clinically it may be difficult to distinguish between primary caveolin-3 deficiency and idiopathic RMD. HyperCKaemia is a well known presentation of mutations in the CAV3 gene. Recently it has even been described in caveolinopathy cases with normal caveolin expression in the muscle [25]. CK elevation can be mild and does correlate neither with the affected caveolin domain nor with disease duration.

Muscle biopsy analysis can be helpful in the diagnosis of caveolinopathy. Reduction or complete loss of caveolin-3 expression was found in 4 biopsies. On the other hand secondary loss of caveolin-3 expression can be seen in other muscle diseases such as LGMD2B [26]. In several patients with caveolin-3-deficiency we excluded a primary CAV3 mutation, but have not been able to identify the underlying cause for the disease. In a recent report, two cases with hyperCKaemia and proven CAV3 mutations showed normal caveolin expression in their muscles [25]. The broad spectrum of clinical phenotypes associated with the same mutation in our cohort of patients confirms the previously mentioned phenotypic variability in caveolinopathy.

In conclusion, this is the first report of a cohort of patients with caveolinopathy in the UK. The phenotypic spectrum seen in caveolinopathy included muscle pain, absence of weakness and myoglobinuria. The differential diagnosis for these symptoms is broad and includes metabolic myopathies, Becker muscular dystrophy and patients with FKRP mutations [27]. Other non-muscle related symptoms such as hypoglycaemia may also be associated with caveolin-3 deficiency. The presence of PIRCs and rippling movements does not seem to impact on the quality of life of patients but is a useful symptom to diagnose caveolinopathy.
**Acknowledgement**

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Figure and table legends

**Figure 1**: Skeletal muscle sections from control muscle (a), patient 1 (b), patient 2 (c) and patient 10 (d) were stained with caveolin-3 antibodies. Caveolin-3 expression was either severely reduced (b) or completely lost (c, d). Magnification 20x for (b) and 10x for (a, c and d). No caveolin-3 band was detectable on the Western blot of patient 1(e). The bands for the other proteins were normal compared to control.
Table 1: The table summarizes the clinical data of the 10 patients.

<table>
<thead>
<tr>
<th>Number</th>
<th>Sex</th>
<th>Age at onset</th>
<th>Presenting symptoms</th>
<th>Age at assessment</th>
<th>Rippling</th>
<th>PIRCs</th>
<th>Weakness</th>
<th>Muscle state</th>
<th>Pain</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>5-6 years</td>
<td>tip toe walking</td>
<td>8 years</td>
<td>yes</td>
<td>yes</td>
<td>proximal upper &amp; lower limbs</td>
<td>mild hypertrophy</td>
<td>no</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>6-7 years</td>
<td>pain &amp; rippling</td>
<td>22 years</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>generalised hypertrophy</td>
<td>yes</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>15 years</td>
<td>cramps &amp; rippling</td>
<td>28 years</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>mild hypertrophy</td>
<td>yes</td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>6-7 years</td>
<td>tip toe walking &amp; large calves</td>
<td>38 years</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>mild hypertrophy</td>
<td>yes</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>15 years</td>
<td>weakness</td>
<td>56 years</td>
<td>no</td>
<td>no</td>
<td>proximal &amp; distal upper &amp; lower limbs</td>
<td>mild hypertrophy of calves &amp; wasting of hand muscles</td>
<td>no</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>7-8 years</td>
<td>rippling</td>
<td>25 years</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td>mild hypertrophy</td>
<td>no</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>7-8 years</td>
<td>tip toe walking &amp; waddling gait</td>
<td>23 years</td>
<td>yes</td>
<td>yes</td>
<td>proximal upper &amp; lower limbs</td>
<td>mild hypertrophy</td>
<td>no</td>
</tr>
<tr>
<td>8</td>
<td>F</td>
<td>30 years</td>
<td>pain</td>
<td>34 years</td>
<td>yes</td>
<td>no</td>
<td>proximal upper &amp; lower limbs</td>
<td>hypertrophy</td>
<td>yes</td>
</tr>
<tr>
<td>9</td>
<td>F</td>
<td>32 years</td>
<td>pain</td>
<td>36 years</td>
<td>yes</td>
<td>yes</td>
<td>proximal &amp; distal upper &amp; lower limbs</td>
<td>hypertrophy</td>
<td>yes</td>
</tr>
<tr>
<td>10</td>
<td>F</td>
<td>13 years</td>
<td>pain on exercise</td>
<td>15 years</td>
<td>no</td>
<td>yes</td>
<td>proximal upper &amp; lower limbs</td>
<td>mild hypertrophy</td>
<td>yes</td>
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</tbody>
</table>
**Table 2:** The table summarizes the results of additional investigations in the 10 patients. AB, antibody; Ck, creatine kinase; EMG, electromyography; * refers to newly described mutations.

<table>
<thead>
<tr>
<th>Patient</th>
<th>CK (age)</th>
<th>EMG</th>
<th>Biopsy</th>
<th>Immunohistochemistry</th>
<th>WB</th>
<th>Mutation</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Caveolin-3</td>
<td>other AB</td>
<td>Caveolin-3</td>
</tr>
<tr>
<td>1</td>
<td>1340 (8y)</td>
<td>myopathic features</td>
<td>normal</td>
<td>absent</td>
<td>no abnormalities</td>
<td>absent</td>
</tr>
<tr>
<td>2</td>
<td>5000 (22)</td>
<td>myopathic features, spontaneous activities at rest</td>
<td>mild fibre size variation</td>
<td>markedly reduced</td>
<td>weak dysferlin expression</td>
<td>absent</td>
</tr>
<tr>
<td>3</td>
<td>1070 (28)</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
</tr>
<tr>
<td>4</td>
<td>1754 (38)</td>
<td>chronic myopathy</td>
<td>dystrophic features</td>
<td>not done</td>
<td>no abnormalities</td>
<td>not done</td>
</tr>
<tr>
<td>5</td>
<td>2316 (56)</td>
<td>not done</td>
<td>dystrophic features and rimmed vacuoles</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
</tr>
<tr>
<td>6</td>
<td>2782 (25)</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
</tr>
<tr>
<td>7</td>
<td>1400 (23)</td>
<td>myopathic features</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
</tr>
<tr>
<td>8</td>
<td>1856 (35)</td>
<td>mild myopathic, spontaneous activities</td>
<td>myopathic changes</td>
<td>absent</td>
<td>weak and patchy beta-spectrin expression</td>
<td>absent</td>
</tr>
<tr>
<td>9</td>
<td>662 (32)</td>
<td>myopathic features</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
<td>not done</td>
</tr>
<tr>
<td>10</td>
<td>887 (15)</td>
<td>not done</td>
<td>dystrophic features</td>
<td>absent</td>
<td>reduced and patchy laminin beta1 &amp; dysferlin expression</td>
<td>not done</td>
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References


