Myotilinopathy: refining the clinical and myopathological phenotype

Montse Olivé,1 Lev G. Goldfarb,2 Alexey Shatunov,2 Dirk Fischer3 and Isidro Ferrer1

1Institut de Neuropatologia, IDIBELL-Hospital de Bellvitge, Hospitalet de Llobregat, Barcelona, Spain, 2The National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892-1361, USA and 3Muskellabor, Department of Neurology, University of Bonn, 53105 Bonn, Germany

Correspondence to: Dr Montse Olivé, Institut de Neuropatologia, IDIBELL-Hospital de Bellvitge, C/Feixa Llarga s/n, 08907 Hospitalet de Llobregat, Barcelona, Spain
E-mail: 25169mop@comb.es

Mutations in myotilin gene (MYOT) have been associated with variable syndromes including limb girdle muscular dystrophy type 1A (LGMD1A) and a subgroup of myofibrillar myopathy (MFM/MYOT). We studied six Spanish patients from three unrelated kindreds and seven patients without family history. Three previously reported and two novel disease-associated MYOT mutations were identified in this group of patients. The disease is characterized by the onset at the age of 42–77 years with muscle weakness initially in distal or proximal leg muscles, eventually spreading to other muscle groups of the lower and upper extremities. Associated signs of cardiomyopathy, respiratory failure and peripheral neuropathy are present in a fraction of patients. Myopathological features of focal myofibrillar destruction resulting in intracytoplasmic deposits, strongly immunoreactive to myotilin, multiple rimmed and centrally or subsarcolemmally located non-rimmed vacuoles and streaming Z-lines, were observed in each patient studied. The Spanish cohort, the largest group of patients studied so far, shares phenotypic features with both LGMD1A and MFM/MYOT variants thus establishing a continuum of phenotypic manifestations characteristic of myotilinopathy, an emerging neuromuscular disorder.

Keywords: LGMD1A; myofibrillar myopathy; myotilin; MYOT mutations; phenotype

Abbreviations: CK = creatinine kinase; EKG = electrocardiography; EMG = electromyography; IBM = inclusion body myositis; LGMD1A = limb girdle muscular dystrophy type 1A; MFM = myofibrillar myopathy; MYOT = myotilin gene; MRC = Medical Research Council; MHC = major histocompatibility complex; RBBB = right bundle branch block; ZASP = Z-band alternatively spliced PDZ motif-containing protein

Received February 9, 2005. Revised April 27, 2005. Accepted May 20, 2005. Advance Access publication June 9, 2005

Introduction

Myotilin is a 57 kDa sarcomeric Z-line associated protein expressed strongly in skeletal muscle and weakly in cardiac muscle (Salmikangas et al., 1999). Myotilin plays a significant role in sarcomere assembly by acting together with α-actinin and filamin C to cross link actin into tightly packed bundles (Salmikangas et al., 2003). The resulting structures support the integrity of the contracting muscle cell (van der Ven et al., 2000). Myotilin is encoded by a single copy gene (MYOT, TTID) located on chromosome 5q31 (Salmikangas et al., 1999; van der Ven et al., 2000; Yamaoka et al., 1994), which encompasses 10 exons and codes for 498 amino acids. The gene is highly conserved among vertebrate species.

Currently known disorders associated with mutations in the myotilin gene (MYOT) (myotilinopathies) include autosomal dominant limb girdle muscular dystrophy type 1A (LGMD1A) (Hauser et al., 2000, 2002) and a subgroup of myofibrillar myopathy (MFM) associated with myotilin mutations (MFM/MYOT) (Selcen and Engel, 2004). LGMD1A has so far been characterized in two families. The patients of a large West Virginian family carrying the myotilin Thr57Ile mutation exhibited an adult-onset proximal leg weakness subsequently spreading to involve distal leg and both proximal and distal arm muscles (Gilchrist et al., 1988; Hauser et al., 2000). The pattern of inheritance was autosomal dominant. Some affected individuals had a
distinctive nasal dysarthric pattern of speech. Myopathic- logical examination demonstrated variation in fibre size, fibre splitting, large number of rimmed vacuoles and patches of Z-line streaming (Gilchrist et al., 1988; Hauser et al., 2000). Members of the second three-generation Argentinean LGMD1A family showing a myotilin Ser55Phe mutation developed proximal limb weakness by age 42–58 years that later progressed to involve distal limb muscles (Hauser et al., 2002). Two of four affected individuals had dysarthric speech. MFM is defined myopathologically as a focal dissolution of the myofilbrils, accumulation of the products of myofilibrillar degradation and ectopic expression of multiple proteins (De Bleecker et al., 1996; Nakano et al., 1996). The highly heterogeneous MFM entity includes subgroups of patients showing mutations in desmin, αB-crystallin, selenoprotein N, Z-band alternatively spliced PDZ motif-containing protein (ZASP) and myotilin (Goldfarb et al., 1996; Vicart et al., 1996; Ferriero et al., 2004; Selcen and Engel, 2004, 2005). The MFM/MYOT subtype was described in six unrelated patients with Ser55Phe, Ser60Cys, Ser60Phe and Ser95Ile myotilin mutations (Selcen and Engel, 2004). The pattern of inheritance was identified as autosomal dominant in one family. The age of disease onset varied from 53 to 77 years. Three patients had muscle weakness in distal leg muscles and two had predominant proximal weakness. Each patient showed clinical, EMG and histologic evidence of peripheral neuropathy on muscle biopsies. Cardiac involvement was detected in three patients. Granular and hyaline deposits, some of them congophilic, and small vacuoles containing membranous material were seen in trichrome stained sections. Strong immunoreactivity for myotilin, dystrophin, desmin, plectin and gelsolin was present. Streaming of the Z-line at the ultrastructural examination was a prominent feature (Selcen and Engel, 2004).

We report on a series of newly identified Spanish patients with myotilinopathy showing a clinical/pathological phenotype that appears to be intermediate between the previously described LGMD1A and MFM/MYOT subtypes, allowing to establish a continuum of clinical/pathological manifestations that define better diagnostic pattern of myotilinopathy.

**Patients and methods**

**Patient and family ascertainment**

We have identified and clinically evaluated 13 patients aged 42–77 years at the disease onset, with 6 patients originating from 3 multigenerational Spanish families (Fig. 1) and additional 7 patients having no family history. All these individuals were a part of a group of 22 patients (13 familial and 9 sporadic cases) who were diagnosed as suffering from MFM based on a muscle biopsy study. Each of the 22 patients was analysed for the presence of mutations in desmin and αB-crystallin genes; 3 of these patients showed mutations in desmin, and no changes were found in αB-crystallin. The following investigations were carried out: pedigree analysis, neurological examination, including muscle strength evaluation according to the Medical Research Council (MRC) grading scale, serum creatinine kinase (CK) level assessment, electrophysiological studies that consisted of sensory (median and sural nerves) and motor (common peroneal nerve) nerve conduction tests and concentric needle EMG (in 11 patients), cardiologic examination with electrocardiography (EKG) and echocardiography, respiratory function tests and muscle CT scanning at midthigh and midlower leg levels (in 7 patients).

**Muscle biopsy**

Open muscle biopsy was performed on 12 patients. Muscle samples were obtained from the soleus muscle in one patient, the quadriceps in four patients, deltoid in four patients and gastrocnemius in the remaining three. In three patients, two muscle biopsies were necessary before the diagnosis of MFM could be established. Samples were processed for routine histochecmical tests and immunocytochemical analysis of desmin, actin, dystrophin, gelsolin, αB-crystallin, ubiquitin, phospho-tau and amyloid βA4 as previously described (Olive et al., 2004). Myotilin immunocytochemistry was carried out using a mouse monoclonal anti-myotilin antibody (Novocastra, Newcastle upon Tyne, UK) at a dilution of 1:150. Immunohistochemical typing of the mononuclear cells and MHC class I immunohistochemistry was performed as described (Ferreer et al., 2004). Amyloid-like deposits were visualized with Congo red. A sample of biopsy tissue was processed for ultrastructural examination using standard methods.

**Single- and double-labeling immunofluorescence analysis and confocal microscopy**

Cryostat sections were stained with saturated solution of Sudan black B (Merck, Darmstadt, Denmark) for 30 min to block autofluorescence of lipofuscin granules, rinsed in 70% ethanol and washed in distilled water. The sections were incubated at 4°C overnight with mouse monoclonal anti-myotilin antibody (Novocastra) at 1:150 dilution and either rabbit polyclonal anti-ubiquitin (Dako, Barcelona, Spain) or goat anti-clusterin antibody (Chemicon, Temecula, CA) at dilutions of 1:100 or 1:200, in a vehicle solution composed of Tris buffer, pH 7.2, containing 15 mmol/l Na3, and protein (Dako). Secondary antibodies were Alexa488 anti-goat and Alexa546 anti-mouse or anti-rabbit (all from Molecular Probes, Eugene, OR) at 1:400 dilution. After washing with PBS, the sections were incubated in a cocktail of secondary antibodies in the same vehicle solution for 45 min at room temperature. After washing in PBS, the sections were mounted in immuno-Fluore Mounting medium (ICN Biomedicals, Costa Mesa, CA), sealed and dried overnight. The sections were examined under a Leica TCS-SL confocal microscope. Sections incubated with just one primary antibody and the corresponding secondary antibody, and sections incubated with the secondary antibodies alone, served as controls.

**Genetic analysis**

Genetic studies were approved by the Institutional Review Board of the National Institute of Neurological Disorders and Stroke, NIH. Genomic DNA was extracted from anticoagulated blood using the Wizard™ Genomic DNA Purification Kit (Promega) and used as template for amplification of MYOT exons with intronic primers designed for this purpose (primer sequences are available on request). Amplification was carried out using an optimal procedure designed for each separate segment. PCR-produced DNA was purified using QIAquick PCR Purification Kit (Qiagen, Valencia, CA) and sequenced in both directions using Dye Terminator™ Sequencing Protocol on an ABI 3100 DNA Analyser (Applied
Biosystems, Foster City, CA). In addition, 100 Spanish unrelated control individuals were analysed for sequence variations in exon 2 of MYOT.

**Results**

**Analysis of gene sequences**

Analysis of MYOT sequences led to the identification of heterozygous missense mutations in 13 patients. The Ser60Phe mutation was detected in one sporadic patient, Ser55Phe in three unrelated sporadic cases and in a multiplex family; the Ser60Cys mutation was identified in one sporadic patient and two other multiplex families. Previously unknown mutations were identified in two patients: an A→G change in codon 36 resulting in a predicted amino acid replacement of lysine with glutamic acid (Lys36Glu), and a C→A alteration at codon 74 that resulted in a glutamine to lysine substitution (Gln74Lys) (Table 1). A single unaffected Spanish individual (a family member of a patient with desminopathy) showed a G→A substitution at codon 115 also resulting in amino acid replacement of threonine for alanine. All these changes were found in the serine-rich area of MYOT that is highly

---

**Table 1** Myotilin mutations

<table>
<thead>
<tr>
<th>Protein domain</th>
<th>Codon</th>
<th>Wild-type codon sequence</th>
<th>Mutant codon sequence</th>
<th>Wild-type amino acid</th>
<th>Mutant amino acid</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine-rich</td>
<td>36</td>
<td>AAA</td>
<td>GAA</td>
<td>Lys</td>
<td>Glu</td>
<td>This study</td>
</tr>
<tr>
<td>Serine-rich</td>
<td>55</td>
<td>TCC</td>
<td>TTC</td>
<td>Ser</td>
<td>Phe</td>
<td>Hauser et al., 2002</td>
</tr>
<tr>
<td>Serine-rich</td>
<td>57</td>
<td>ACA</td>
<td>ATA</td>
<td>Thr</td>
<td>Ile</td>
<td>Hauser et al., 2000</td>
</tr>
<tr>
<td>Serine-rich</td>
<td>60</td>
<td>TCT</td>
<td>TGT</td>
<td>Ser</td>
<td>Cys</td>
<td>Selcen and Engel, 2004</td>
</tr>
<tr>
<td>Serine-rich</td>
<td>60</td>
<td>TCT</td>
<td>TTT</td>
<td>Ser</td>
<td>Phe</td>
<td>Selcen and Engel, 2004</td>
</tr>
<tr>
<td>Serine-rich</td>
<td>74</td>
<td>CAG</td>
<td>AAG</td>
<td>Gln</td>
<td>Lys</td>
<td>This study</td>
</tr>
<tr>
<td>Serine-rich</td>
<td>95</td>
<td>AGC</td>
<td>ATC</td>
<td>Ser</td>
<td>Ile</td>
<td>Selcen and Engel, 2004</td>
</tr>
</tbody>
</table>
conserved through the evolution (Hauser et al., 2000). There were no irregularities in this region on sequencing of 100 (200 chromosomes) Spanish control individuals.

**Clinical features**

The type of inheritance was autosomal dominant in two families with five studied patients, whereas in the other seven patients there was no evidence of a positive family history in at least three generations. The remaining patient had a brother similarly affected, but the pattern of inheritance could not be established. Detailed clinical examination of non-symptomatic first-degree relatives of two sporadic cases detected no signs of myopathy.

**Family 1**

Of eight members known to be affected, a father and his two sons (II:6, III:1 and III:2, Fig. 1) showing the myotilin heterozygous Ser55Phe mutation were studied in detail. They developed weakness in distal muscles of the legs at the age of 48–50 years progressing to involve other limb muscles and neck extensors. On examination, the father and the older son had severe weakness and wasting of distal leg muscles and to a lesser extend hip adductors, knee flexors, iliopsoas, deltoids, wrist and finger extensors and neck extensors (Fig. 2). Deep tendon reflexes were absent in the lower limbs. Speech was not affected and sensation was intact. CK levels were twice the normal values. EKG and echocardiography examinations were normal. EMG showed signs of myopathy with spontaneous activity at rest. Nerve conduction studies were normal. The younger son had similar but milder symptoms. Other family members presented around the age of 50 years with weakness in the distal leg muscles eventually spreading to other muscle groups. None had dysarthria or cardiac involvement.

**Family 2**

Six members of another Spanish family suffered from muscle weakness and cardiomyopathy. A father and daughter (II:2 and III:2, Fig. 1) carrying the Ser60Cys myotilin mutation presented at ages 60 and 58 years with bilateral foot drop eventually involving proximal leg muscles and the upper limbs. Examination revealed severe weakness and wasting of the anterior and posterior leg compartments, hip adductors, knee flexors as well as wasting of deltoids, wrist and finger extensors, more pronounced in the father. Reflexes were absent or diminished throughout. Speech was not affected and sensation was intact. At the age of 70 years, the father was diagnosed with cardiomyopathy and died from congestive cardiac failure. The daughter developed cardiomyopathy at the age of 72 years; her echocardiographic study showed mild hypokinesia of the right ventricle with no overt hypertrophy or dilatation of the heart chambers. Respiratory function tests did not show any abnormalities. Serum CK was within normal levels. Four other family members had a similar disease and three died of cardiac failure.

**Family 3**

An 81-year-old man, carrying the Ser60Cys myotilin mutation, presented at the age of 65 years with weakness in the distal leg muscles later involving proximal muscles of lower limbs and upper extremities. Examination revealed hypernasal voice, and severe weakness and wasting of the anterior and posterior leg muscles, iliopsoas, hip adductors, knee flexors as well as wasting of deltoids, wrist and finger extensors. Reflexes were hypoactive throughout; heel cords were tight. At the age of 70 years, he developed restrictive respiratory insufficiency that required nocturnal ventilation support. CK levels were at normal values. EKG showed a left bundle branch block (LBBB). Echocardiography was normal. EMG showed signs of myopathy with spontaneous activity at rest. Nerve conduction studies were normal. His only brother was similarly affected; both parents had died with no signs of myopathy.

**Sporadic cases**

Of seven sporadic patients, three presented with weakness in the distal leg muscles, two others had initial weakness in proximal muscles of the lower limbs and one patient presented with proximal and distal leg weakness. In the advanced phases of illness, all patients had severe weakness in distal and proximal leg muscles, and mild to moderate weakness in deltoids and wrist extensors. None had speech disturbances. Deep tendon reflexes were absent at the ankle and knee, but commonly preserved in the upper extremities. Tight heel cords were noted in three patients. A 78-year-old male had hypesthesia to all modalities; his nerve conduction studies suggested a mild motor and sensory axonal neuropathy. At the age of 75 years, this patient developed severe respiratory insufficiency that required nocturnal ventilation support. EKG showed a left bundle branch block in this patient.
No respiratory insufficiency, cardiomyopathy or signs of neuropathy were observed in other patients in this group. CK level was normal or slightly elevated. Needle EMG showed signs of myopathy with spontaneous activity at rest. The remaining patient presented at the age of 42 years with dyspnea on exertion, later progressing to severe respiratory insufficiency. The patient also developed distinct nasal voice and mild muscle weakness in iliopsoas, knee flexors, hip adductors, wrists...
Fig. 4 Histochemical findings in myotilinopathy patients. (A) Variation in the fibre size, increased numbers of internal nuclei, abnormal inclusions in the cytoplasm or under the sarcolemma; a large rimmed vacuole is seen in an atrophic fibre. (B) Giant fibre containing hyaline deposits and multiple vacuoles. (C) Several rimmed vacuoles in a single fibre. (D) Rimmed and non-rimmed vacuoles and eosinophilic inclusions in a muscle fibre; an adjacent small fibre also contains non-rimmed vacuoles. (E) Mononuclear cells invading an apparently normal fibre. (F) Muscle fibre necrosis with phagocytosis and small regenerating fibres. A hyaline inclusion in the adjacent fibre. (G) Several abnormal fibres containing blue–red amorphous inclusions and vacuoles. (H) A small cluster of cytoplasmic bodies (top), and a serpentine hyaline structure (bottom). (I) Group of fibres bearing hyaline inclusions and small red spheroïd bodies. (J) Well delimitated areas lacking oxidative enzyme activity. (K) Strong PAS positivity. (L) PAS positivity removed by diastase digestion. A–F: H and E; G–I: modified trichrome; J: NADH; K: PAS stain; L: PAS-diastase. A, C, F, H: Family 1, younger son; B, D, E, J–L: sporadic case with Ser60Cys mutation; G: Family 2, father; I: sporadic case with Ser60Cys mutation. Bar in A = 25 μm; B–L, bar in L = 25 μm.
and finger extensors. In addition, there was weakness and atrophy of pectoralis and sternocleidomastoid muscles. EKG showed an RBBB. Echocardiographic study was normal.

The disease progressed very slowly; 4 of 12 patients became wheelchair-dependent 10–20 years after the disease onset.

**CT-scan studies**

In patients with initial distal leg weakness, muscle CT scans at the midcalf level showed fatty replacement in soleus and medial gastrocnemius muscles followed by anterior tibialis and peroneal group, and mild involvement of the hip adductors (Fig. 3). In cases with proximal onset, muscle hypodensity was noted in the hip adductors, biceps femoris, semimembranosus and sartorius with no obvious changes in the calf muscles. Finally, in the patient with both proximal and distal initial muscle weakness, marked decrease of attenuation was observed in medial gastrocnemius, soleus, hip adductors and biceps femoris. In advanced illness, all muscles of the anterior and posterior compartments of the legs were completely replaced by fatty tissue, irrespective of the mode of presentation. The quadriceps muscles, especially rectus femoris and vastus lateralis, were relatively well preserved.

**Histologic features**

Muscle biopsy in 11 affected individuals showed marked variation in fibre size and increased number of internal nuclei. Atrophic rounded fibres of either histochemical type often clustered in small groups (Fig. 4A). Polymorphic inclusions of varying size and shape (Fig. 4A, B, D, G–I) were seen in multiple muscle fibres of 11 patients. These inclusions appear as non-hyaline irregular patches located in the centre of the fibre or in the subsarcolemmal space (Fig. 4A). They appear pink with HE and dark-blue with the modified trichrome Gomori stain. Single or multiple hyaline inclusions staining bright pink with HE (Fig. 4B and D) and blue–red or red–purple with the modified trichrome Gomori stain.

![Fig. 5](https://academic.oup.com/brain/article-abstract/128/10/2315/274639)
(Fig. 4G–I) were also observed; they varied in shape and size and lacked oxidative (Fig. 4J) and ATPase activity. Some hyaline structures were congophilic.

Large numbers of lobulated fibres on oxidative stains and fibre type grouping were noted in a single patient. Cytoplasmic bodies were seen in most cases (Fig. 4H and I). Single or multiple vacuoles rimmed by basophilic membranous material (Fig. 4A and C) were observed in each patient studied. Large numbers of non-rimmed vacuoles (Fig. 4B, D, G and I) were observed in nine cases. The non-rimmed vacuoles appeared as empty spaces located under the sarcolemma or in the cytoplasm. Some of them showed strong PAS-positivity that disappeared after diastase digestion (Fig. 4K and L). Strong acid phosphatase activity was noted in some fibres. Foci of mononuclear inflammatory cells (Fig. 4E and F), mainly composed of CD8$^+$T cells invading necrotic and non-necrotic fibres were prominent in four cases. Sarcolemmal expression of MHC class I antigens was not observed. The morphological abnormalities tended to be restricted to certain areas of the biopsy specimen and no abnormality was observed in muscles showing no signs of weakness.

Immunohistochemical studies showed strong immunoreactivity for myotilin, desmin, αB-crystallin, ubiquitin, phospho-tau, dystrophin, actin, gelsolin, amyloid βA4 in the abnormal fibres of each studied patient (Fig. 5A–I). Under confocal microscopy, myotilin deposits appeared as confluent patches covering significant parts of the muscle fibre or as multiple aggregates scattered in the cytoplasm or subsarcolemmal spaces. Atrophic fibres displayed diffuse myotilin immunoreactivity (Figs 6 and 7). Myotilin largely co-localized with ubiquitin (Fig. 6) and clusterin (Fig. 7) as revealed by double-labelling immunofluorescence.

On electron microscopy, streaming of the Z-line and focal dissolution of myofibrils was noted in each studied case. Electron-dense structures corresponding to the remnants of the Z-lines were always present. Large numbers of autophagic vacuoles and myelin-like structures were present in each case studied (Fig. 8). In addition, affected fibres contained large amounts of granular material and free glycogen.
Discussion

Thirteen newly identified patients showing mutations in the myotilin gene were studied and the results compared with previous reports describing two other groups of patients with myotilinopathy (Table 2). The pattern of inheritance was autosomal dominant in LGMD 1A, but some patients of the MFM/MYOT group and our group did not have family history of disease. The age of disease onset in our patients (42–77 years) is overlapping with both LGMD1A and MFM/MYOT cohorts. The rate of disease progression was very slow, taking >10 years before the patients became disabled. Distal muscle weakness of the lower limbs was the presenting symptom in 10 patients, whereas proximal leg weakness was the initial complaint in 2. A similar ratio was observed in the MFM/MYOT group (Selcen and Engel, 2004), but all LGMD1A patients had proximal leg weakness (Gilchrist et al., 1988). In a single patient, respiratory insufficiency was the first manifestation of the disease. With the progression of illness, all limb muscles were affected in each group of patients leading to quadriplegia at an advanced stage. In the majority of our patients, foot drop owing to anterior tibialis weakness was the first symptom; however, CT-scan studies performed early in the course of illness suggested that the disease actually started in the posterior lower leg muscles with no significant functional consequences until the anterior group became weak. As the disease progressed, iliopsoas, knee flexors and hip adductors became weak. In patients with distal weakness at the onset, involvement of the upper extremities occurred 5–7 years after the disease onset. Wrist and finger extensors and deltoids were the most affected muscles. Weakness of neck extensors was noted in some patients of either group. Facial muscles were affected in 3 of 16 patients of the LGMD1A original family, but have not been seen in other groups. In a single patient with the Gln74Lys mutation pectoralis muscles were affected.

Some patients in both LGMD1A families had dysarthric speech, whereas the MFM/MYOT did not have dysarthria and only two of the patients we studied had hypernasal voice. Deep tendon reflexes were decreased at the ankles in patients of either group, but in most cases were present in the arms. The tightness of Achilles tendons, a characteristic feature in the LGMD1A families, was rarely present in the MFM/MYOT

Fig. 7 Double-labelling immunofluorescence reactions with clusterin (green, A, D) and myotilin (red, B, E) in abnormal muscle fibres. Note the co-localization of clusterin and myotilin (merge yellow, C, F) (sporadic case with Ser60Cys mutation). Sections immunostained with the secondary antibodies alone served as negative controls (G, H and I).
Peripheral neuropathy was a uniform feature in the MFM/MYOT group; in view of this fact, we performed motor and sensory nerve conduction studies which were normal in each patient studied, except for a single patient with distal hypesthesia who showed a symmetric motor and sensory axonal polyneuropathy. Peripheral neuropathy was also absent in patients with the LGMD1A phenotype (Gilchrist et al., 1988).

Signs of cardiomyopathy were present in the members of the Ser60Cys family, and EKG abnormalities without advanced heart block or cardiac dysfunction were observed in three unrelated patients. Cardiac involvement has previously been reported in three MFM/MYOT patients but not in patients with the LGMD1A phenotype. It has been suggested that the absence of cardiac dysfunction in LGMD1A patients could be related to the younger age of these patients at the time of examination (Selcen and Engel, 2004), however some of our patients were examined at the age of 78–82 years and showed no evidence of cardiomyopathy. These observations suggest that involvement of cardiac muscle is not a constant feature of myotilinopathy. Three patients in our group developed restrictive respiratory failure with severely reduced forced vital capacity necessitating nocturnal ventilatory support. This is the first report of respiratory insufficiency in patients with myotilinopathy.

Histologic characteristics make an especially strong contribution to the phenotypic definitions of myotilinopathy. Hyaline and non-hyaline lesions were observed in the majority of the patients we studied and the patients of the MFM/MYOT group (Selcen and Engel, 2004), but were not reported in the LGMD1A patients. Rimmed vacuoles corresponding to the autophagic vesicles observed under EM were present in patients of each group. In addition to the rimmed vacuoles, single or multiple non-rimmed vacuoles were also observed. These vacuolated areas represent foci of myofibrillar destruction containing remnants of degraded myofibrillar structures, membranous material and glycogen granules; therefore, they should be viewed as cytoplasmic spaces rather than true vacuoles. The striking vacuolar changes are important clues strongly indicating myotilinopathy. Z-line irregularity found in each group is another unifying feature of myotilinopathy. We observed focal inflammatory changes in four patients and similar changes were previously described in a single MFM/MYOT patient. Abnormal protein deposits found in our group of patients were similar to those reported in patients with other types of MFM (De Bleecker et al., 1996; Olivé et al., 2004; Ferrer et al., 2004; Selcen et al., 2004).

The causes and mechanisms of protein aggregation in myotilinopathy patients remain unknown. We recently suggested that impaired proteasomal expression may explain in part the abnormal protein accumulation (Ferrer et al., 2004). In the present study, clusterin depositions co-localizing with myotilin aggregates were found in many abnormal fibres.
Clusterin (Apolipoprotein J, SP40,40), a sialoglycoprotein with a nearly ubiquitous tissue distribution (Jones et al., 2002), is found as a component of abnormal protein accumulation in several neurodegenerative diseases including β-amyloid plaques in Alzheimer’s disease (McGeer et al., 1992; Lidström et al., 1998), prionopathies (Sasaki et al., 2002a; Freixes et al., 2004) and α-synucleinopathies (Sasaki et al., 2002b). The results of the present study suggest that clusterin participates in protein aggregation occurring in myotilinopathy patients. Clusterin expression and the aggresome in MFM and other myopathic conditions are analysed in detail elsewhere (Ferrer et al., 2005).

Inflammatory changes in late onset distal myopathy with mild or no CK elevation, myopathic EMG with prominent spontaneous activity, rimmed vacuoles and protein deposits in muscle fibres may be confused with inclusion body myositis (IBM). Early involvement of quadriceps and finger flexors in IBM, which are relatively spared in myotilinopathies, are important clinical clues for the diagnosis. Furthermore, in contrast to IBM, the expression of MHC class I antigens in the cytoplasmic membrane is not observed.

Of the entire group of 22 patients we had the opportunity to study, the pathologic features of 13 myotilinopathy patients differed from those in 3 patients with desminopathy and 6 genetically unidentified patients in several respects:

(i) The myofibrillar lesions observed in desminopathy cases and genetically unidentified cases were less abundant, less florid and mainly observed as discrete plaque-like inclusions compared with irregular polymorphous inclusions observed in myotilinopathy.

(ii) Vacuolar changes were much more prominent in myotilinopathy patients.

(iii) The granulofilamentous material typically observed in desminopathies at the ultrastructural level was not observed in cases of myotilinopathy.

### Table 2 Phenotypic characteristics of myotilinopathies

<table>
<thead>
<tr>
<th>Phenotypic features</th>
<th>LGMD1A original family*</th>
<th>LGMD1A second family†</th>
<th>MFMI/MYOT familial and sporadic patients‡</th>
<th>Present study</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of studied patients</td>
<td>16</td>
<td>4</td>
<td>6</td>
<td>13</td>
</tr>
<tr>
<td>Myotilin mutation</td>
<td>Thr57Ile</td>
<td>Ser55Phe</td>
<td>Ser55Phe, Ser60Cys, Ser60Phe, Ser95Ile</td>
<td>Lys36Glu, Ser55Phe, Ser60Cys, Ser60Phe, Gln74Lys</td>
</tr>
<tr>
<td>Pattern of inheritance</td>
<td>AD</td>
<td>AD</td>
<td>AD, sporadic</td>
<td>AD, sporadic</td>
</tr>
<tr>
<td>Onset age</td>
<td>18–35</td>
<td>42–58</td>
<td>50–77</td>
<td>47–77</td>
</tr>
<tr>
<td>Rate of progression</td>
<td>Slow</td>
<td>Slow</td>
<td>Slow</td>
<td>Slow</td>
</tr>
<tr>
<td>Muscle weakness at onset</td>
<td>PLL in 13, PUL in 11</td>
<td>PLL, PUL</td>
<td>DPL, PUL</td>
<td>DPL, PUL</td>
</tr>
<tr>
<td>Weakness in additional muscle groups</td>
<td>DUL in 4, facial in 3</td>
<td></td>
<td>DUL</td>
<td>PUL, DUL, neck in 3, pectoralis and sternocleidomastoid in 1</td>
</tr>
<tr>
<td>Muscle atrophy</td>
<td>No</td>
<td>Not reported</td>
<td>2/5</td>
<td>7/13</td>
</tr>
<tr>
<td>Peripheral neuropathy</td>
<td>No</td>
<td>Not reported</td>
<td>6/6</td>
<td>1/13</td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td>0/16</td>
<td>Not reported</td>
<td>3/5</td>
<td>1/13</td>
</tr>
<tr>
<td>Respiratory insufficiency</td>
<td>0/16</td>
<td>Not reported</td>
<td>0/5</td>
<td>3/13</td>
</tr>
<tr>
<td>Muscle stiffness, myalgia</td>
<td>0/16</td>
<td>Not reported</td>
<td>1/6</td>
<td>4/13</td>
</tr>
<tr>
<td>Decreased ankle reflexes</td>
<td>1/16/16</td>
<td>Not reported</td>
<td>4/5</td>
<td>12/13</td>
</tr>
<tr>
<td>Decreased reflexes in the arms</td>
<td>5/16</td>
<td>Not reported</td>
<td>1/5</td>
<td>5/13</td>
</tr>
<tr>
<td>Heel cord contracture</td>
<td>10/16</td>
<td>Not reported</td>
<td>1/5</td>
<td>4/13</td>
</tr>
<tr>
<td>Creatine kinase level</td>
<td>1.6–9-fold increase</td>
<td>5–15-fold increase</td>
<td>Normal in 3/6; 2-fold increase in 3/6</td>
<td>Normal in 9/13; 2-fold increase in 3/13 4-fold increase in 11/13</td>
</tr>
<tr>
<td>EMG</td>
<td>Myopathic</td>
<td>Not reported</td>
<td>Myopathic in 1/6; myopathic and neurogenic in 4/5</td>
<td>Myopathic in 10/11, myopathic and neurogenic in 1/11</td>
</tr>
<tr>
<td>Nerve conduction</td>
<td>Normal</td>
<td>Not reported</td>
<td>Not reported</td>
<td>Not reported</td>
</tr>
<tr>
<td>Myopathology</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrophic fibres</td>
<td>3/8</td>
<td>Not reported</td>
<td>4/6</td>
<td>9/12</td>
</tr>
<tr>
<td>Fibre grouping</td>
<td>No</td>
<td>Not reported</td>
<td>2/6</td>
<td>1/12</td>
</tr>
<tr>
<td>Non-hyaline deposits</td>
<td>Not reported</td>
<td>Not reported</td>
<td>6/6</td>
<td>11/12</td>
</tr>
<tr>
<td>Hyaline deposits</td>
<td>Not reported</td>
<td>Not reported</td>
<td>6/6</td>
<td>11/12</td>
</tr>
<tr>
<td>Rimmed vacuoles</td>
<td>Yes</td>
<td>Not reported</td>
<td>6/6</td>
<td>12/12</td>
</tr>
<tr>
<td>Non-rimmed vacuoles</td>
<td>No</td>
<td>Not reported</td>
<td>No</td>
<td>9/12</td>
</tr>
<tr>
<td>EM: Z-line streaming</td>
<td>Yes</td>
<td>Not reported</td>
<td>6/6</td>
<td>12/12</td>
</tr>
<tr>
<td>Autophagic vacuoles</td>
<td>Yes</td>
<td>Not reported</td>
<td>6/6</td>
<td>12/12</td>
</tr>
</tbody>
</table>

PLL, proximal lower limb; DLL, distal lower limb; PUL, proximal upper limb; DUL, distal upper limb. *Hauser et al., 2000; †Hauser et al., 2002; ‡Selcen and Engel, 2004.
Accumulating data suggest that myotilinopathy constitutes ~10% of MFM patients investigated in large multinational centres (Selcen and Engel, 2004; Lev G, Goldfarb, unpublished data). The frequency of myotilinopathy among patients with MFM in Spain may be much higher. The reasons for the high frequency of myotilinopathy in the Spanish population are under investigation.

In conclusion, our clinical and myopathological data define more precisely the myotilinopathy phenotype. Although the initial weakness could be in distal or proximal lower limb muscles, cardiomyopathy and respiratory insufficiency may be present or absent, the overall clinical and pathological spectrum is recognizable. The phenotypic features of the group of patients described in this report overlap with previously characterized LGMD1A and MFM/MYOT groups.

Acknowledgements
We would like to thank the patients and families for collaboration, the clinicians who sent their patients for evaluation, especially Drs M. Huerta, S. Jaumà, J. A. Martínez-Matos, M. Povedano, A. Pou, E. Farrero and E. Prats. We also wish to thank Drs Alió and F. Martínez from the Cardiology and Radiology Departments for their collaboration, and R. Blanco, M. Carmona, D. Moreno, E. Laforet and B. Torrejón-Escribano for their excellent technical assistance. We would like to thank the clinicians who sent their patients for evaluation, especially Drs M. Huerta, S. Jaumà, J. A. Martínez-Matos, M. Povedano, A. Pou, E. Farrero and E. Prats. We also wish to thank Drs Alió and F. Martínez from the Cardiology and Radiology Departments for their collaboration, and R. Blanco, M. Carmona, D. Moreno, E. Laforet and B. Torrejón-Escribano for their excellent technical assistance. This work was supported in part by FIS grants 02-0005 and C03-006. D.F. was supported by the Deutsche Forschungsgemeinschaft (Fi 913/2-1).

References