

# Limb girdle muscular dystrophy due to *LAMA2* gene mutations: new mutations expand the clinical spectrum of a still challenging diagnosis

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## Conflict of interest

The Authors declare no conflict of interest

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Mutations in *LAMA2* gene, encoding merosin, are generally responsible of a severe congenital-onset muscular dystrophy (CMD type 1A) characterized by severe weakness, merosin absence at muscle analysis and white matter alterations at brain Magnetic Resonance Imaging (MRI). Recently, *LAMA2* mutations have been acknowledged as responsible of LGMD R23, despite only few cases with slowly progressive adult-onset and partial merosin deficiency have been reported. We describe 5 independent Italian subjects presenting with progressive limb girdle muscular weakness, brain white matter abnormalities, merosin deficiency and *LAMA2* gene mutations. We detected 7 different mutations, 6 of which are new. All patients showed normal psychomotor development and slowly progressive weakness with onset spanning from childhood to forties. Creatin-kinase levels were moderately elevated. One patient showed dilated cardiomyopathy. Muscle MRI allowed to evaluate the degree and pattern of muscular involvement in all patients. Brain MRI was fundamental in order to address and/or support the molecular diagnosis, showing typical widespread white matter hyperintensity in T2-weighted sequences. Interestingly these alterations were associated with central nervous system involvement in 3 patients who presented epilepsy and migraine. Muscle biopsy commonly but not necessarily revealed dystrophic features. Western-blot was usually more accurate than immunohistochemical analysis in detecting merosin deficiency. The description of these cases further enlarges the clinical spectrum of *LAMA2*-related disorders. Moreover, it supports the inclusion of LGMD R23 in the new classification of LGMD. The central nervous system involvement was fundamental to address the diagnosis and should be always included in the diagnostic work-up of undiagnosed LGMD.

**Key words:** limb girdle muscular dystrophy, merosin, *LAMA2* gene, brain MRI, muscle MRI, leukoencephalopathy

## Introduction

Laminin-2 (also called merosin), a heterotrimeric protein composed of three different subunits ( $\alpha 2\beta 1\gamma 1$ ), is a major component of the basement membrane of skeletal muscle fibres, Schwann cells in peripheral nerves, brain capillaries and submandibular glands <sup>1</sup>. It belongs to a large family of glycoproteins involved in extracellular matrix architecture, cell adhesion and differentiation and mediates the attachment, migration, and organization of cells into tissues during embryonic development by interacting with other extracellular matrix components <sup>1,2</sup>. Mutations in *LAMA2* gene (chromosome 6q22.33), which encodes alpha 2 chain, have been so far identified as the cause of two different clinical phenotypes: an early-onset congenital muscular dystrophy (CMD), also known as CMD1A (congenital muscular dystrophy 1A), and a milder late-onset limb-girdle type muscular dystrophy (LGMD) phenotype.

CMD1A is one of the most frequent forms of classic CMD in Western countries. This form, commonly associated with complete absence of laminin- $\alpha 2$ , is characterized by severe muscle weakness and hypotonia at birth or in the first six months of life, delayed motor development, inability to walk unsupported, joint contractures, high creatine-kinase (CK) levels and widespread white matter abnormalities on brain magnetic resonance imaging (MRI) <sup>2-4</sup>.

In 1998 Bushby et al. described for the first time a case with adulthood onset and mutations in *LAMA2* gene <sup>5</sup>. These forms are generally characterized by slowly progressive proximal muscular weakness, white matter lesions on MRI and partial protein loss at muscle analysis <sup>6-8</sup>.

Recently the 229<sup>th</sup> ENMC International Workshop revised LGMD nomenclature and classification, providing a precise definition of LGMD and including *LAMA2* gene as causative of LGMD R23 <sup>9</sup>.

A potential correlation between the degree of merosin expression, the genotype and the clinical features has been described <sup>10</sup>. In particular, mutations that lead to partial laminin- $\alpha 2$  deficiency are generally associated to milder phenotypes, which range from mild forms of CMD to adult-onset presentations. Mild CMD forms present a benign evolution and slow course, but are always characterized by delay in motor milestones acquisition <sup>11-14</sup>, while the adult-onset LGMD forms generally do not show symptoms at birth. The adult-onset cases are generally due to missense or in-frame splice-site mutations while CMD cases are more frequently associated to nonsense or out-of-frame mutations.

While the prevalence of MDC1A is estimated to be 0.6/100,000 in Northern England (around 10-30% of all

the MDC) <sup>14-15</sup>, the prevalence of LGMD due to *LAMA2* mutations is unknown. However it is believed to be very rare and since now only few cases with adult LGMD presentation have been reported <sup>6-8,17-22</sup>.

Here we describe a small group of Italian subjects carrying homozygous or compound heterozygous mutations in *LAMA2* gene, who developed mild and slowly progressive muscular weakness with limb girdle muscular dystrophy phenotype.

The description of these cases, which include patients with late onset seizures, very late disease onset as far as marginal muscle involvement, contributes to enlarge the spectrum of *LAMA2* gene disorders. In many of these cases the incidental finding of brain white matter abnormalities was fundamental in order to direct the diagnosis.

## Materials and methods

### *Clinical evaluation*

Patients were selected from a cohort of adult LGMD patients evaluated at IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico of Milan and at Department of Neurosciences of Padua. All patients underwent a systematic clinical characterization, including comprehensive neurological, cardiac (electrocardiogram and echocardiogram) and respiratory (spirometry) assessments. Data about familiar and personal history, in particular about age of onset and progression of symptoms, were collected. Muscle strength was evaluated using the Medical Research Council (MRC) Scale. Electromyography (EMG) was performed at the beginning of the disease. The patients were also studied with both Brain and Muscle Magnetic Resonance Imaging (MRI). In selected cases further studies such as electroencephalography were performed.

Written informed consent was obtained (and preserved in the original form) from all subjects or their caregivers when primary diagnostic procedures were performed, with explicit consent to future uses for research purpose, according to the Declaration of Helsinki.

### *Brain and muscle imaging*

Brain imaging data were obtained in all patients by nuclear Magnetic Resonance Imaging (MRI). A 1.5T scanner was used and spin echo (SE), turbo spin echo (TSE) and fluid attenuated inversion recovery (FLAIR) sequences with T1 and T2 weighted images in the three orthogonal planes were acquired.

Muscle MRI was performed using a 1.5T scanner, axial TSE T1 weighted- images and axial short tau inversion (STIR) T2-weighted images were acquired. Both proximal upper and lower limbs' muscles were studied.

### Muscle tissue analysis

All probands underwent muscle biopsy after giving written informed consent, according to Institutional guidelines. Morphological examination was performed according to standard procedures<sup>23</sup>.

The proteins involved in LGMDs were studied using immunohistochemical (IHC) analyses with monoclonal antibodies directed against dystrophin (Novocastra, Newcastle upon Tyne, UK), sarcoglycans (Novocastra, Newcastle upon Tyne, UK), alpha-dystroglycan (a-DG) (Upstate Biotechnology, Lake Placid, NY), and caveolin-3 (Transduction Laboratories, Lexington, KY) as previously described. Calpain-3 and dysferlin expression were analyzed through Western blot (WB) analysis.

Immunostaining of laminin  $\alpha 2$  was performed using two commercially available antibodies directed against the 80-kDa carboxyl-terminus (MAb1922; Chemicon, Temecula, California) and against the amino-terminus (NCL-MER3, Novocastra, Newcastle Upon Tyne, UK). Laminin- $\alpha 2$  immunoblot was performed using the MAb1922 antibody (Chemicon)<sup>24</sup>.

### Molecular analysis

Genomic DNA was extracted from peripheral blood samples according to standard procedures (Flexi Gene DNA Handbook, Qiagen). Molecular analysis of *LAMA2* gene was performed through the amplification by Polymerase Chain reaction (PCR) and direct sequencing (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA) of all the exons and the adjacent intron regions. Primer sequences and PCR conditions are available upon request. Mutations were named according to the Leiden Muscular Dystrophy database (www.dmd.nl). For cDNA numbering, +1 corresponds to the A of the ATG translation initiation codon in the reference sequence.

The pathogenic nature of the mutations was confirmed by screening of 160 control healthy subjects and the parental origin of each mutation was assessed through analysis of parental genomic DNA when available. Furthermore, the amino acid conservation was confirmed by

comparison with the sequence in different species. In few patients, mRNA was isolated from muscle tissue with Eurozol. Then cDNA was produced through reverse transcription polymerase chain reaction (RT-PCR) using the Ready-To-Go RT-PCR kit (Amersham Pharmacia) and analysed by mRNA amplification and sequencing.

In one patient the diagnosis was achieved with Next Generation Sequencing (NGS) techniques and confirmed by Sanger sequencing. DNA samples underwent Motor HaloPlex Target Enrichment protocol, analyzing the coding regions of 89 genes; libraries were run on a HiSeq instrument and analyzed through a custom pipeline of bioinformatics analyses<sup>25</sup>.

## Results

We selected a cohort of 5 patients clinically diagnosed as LGMD and carrying mutations in *LAMA2* gene. We identified 7 different mutation, 6 of which are novel. The molecular, clinical and biophysical features of the patients are summarised in Table I, Table II and Table III.

### Patient I

Patient I was an Italian 30-year-old man without family history for neuromuscular disorders. He presented to medical attention with a LGMD phenotype characterized by progressive limb girdle weakness, predominantly involving lower limbs, and moderate CK levels increase (2-5x; range 454-1000 UI/L). He was the third son of non-consanguineous parents; his parents and his daughter did not show any muscular involvement and had normal CK levels. The patient was born after a normal pregnancy and had a normal psycho-motor development. He presented the first symptoms at 28 years of age when he started to complain difficulty in climbing stairs. The disease had a slowly progressive course with worsening of lower limbs weakness and asymmetrical involvement of proximal upper limb muscles starting from the age of 31 years. Electromyography showed neurogenic alterations without myopathic signs. Clinical evaluation at 59 years of age showed severe prox-

**Table I.** Molecular data showing the two pathogenic mutations and their proteic effect for each patient. Most of the mutations were not previously described in literature.

Pt	Nucleotide 1	Protein 1	Exon 1	Ref.	Nucleotide 2	Protein 2	Exon 2	Ref.
I	c.6742delC	p.Leu2248TrpfsX23	48	New	c.8544C > G	p.His2848Gln	60	New
II.1	c.4005T > C	p.Lys1469Arg	30	New	c.4005T > C	p.Lys1469Arg	30	New
III	c.2750 + 2 insT	p.Glu892Ala del 893_917	IVS19	New	c.2750 + 2 insT	p.Glu892Ala del 893_917	IVS19	New
IV	c.752T > C	p.Leu25Pro	5	New	c.7586_7589dup	p.Phe2531X	55	New
V	c.752T > C	p.Leu25Pro	5	New	c.7147C > T	p.Arg2383X	50	[35]

**Table II.** Clinical data about the patients described.

Pt	I	II.1	III	IV	V
<b>Sex</b>	M	F	M	M	F
<b>Age (yrs)</b>	62	51	75	41	52
<b>Onset (age)</b>	28 y	Childhood	40s	Childhood	Childhood
<b>Symptoms at onset</b>	Proximal weakness	Proximal weakness	Fatigability and distal weakness	Proximal weakness	Proximal weakness
<b>Phenotype</b>	LGMD	LGMD	LGMD	LGMD	LGMD
<b>CK (UI/L)</b>	454-1000	300-400	500-800	677-1640	180-680
<b>Loss of ambulation</b>	No (59 y)	No (51 y)	No (75y)	No (41y)	no (52y)
<b>Joint retractions</b>	No	Mild TT	No	TT and elbows	TT
<b>Sural hypertrophy</b>	Yes	Yes	Yes	No	No
<b>Cardio-pulmonary involvement</b>	BBD	PSVT	Dilated cardiomyopathy, atrial flutter	None	Mild MIP and MEP reduction
<b>Muscle MRI</b>	Severe asymmetric fibro-fatty replacement biceps brachii, triceps brachii, thigh. Selectively sparing of deltoid, sartorius, gracilis, rectus femoris and short head of the biceps femoris	Fatty substitution in trapezius, supraspinatus, subscapularis, infraspinatus and pectoral thigh and leg muscles with relative sparing of the sartorius, gracilis, short head of the biceps femoral and tibialis posterior muscles	Diffuse fibro-fatty substitution of the gluteal muscles, of the posterior thigh, adductor magnus and of the quadriceps, sparing only the rectus femoris. The shoulder muscles were mild affected	NA	Moderate fatty substitution of scapular girdle muscles, severe substitution of pelvic girdle and thigh muscles, with sparing of sartorius, tensor fasciae latae, obturator and iliopsoas, gracilis, biceps femoris and adductor longus on the right side (52 years)
<b>Brain MRI</b>	WMA (U fibres sparing)	WMA	WMA	WMA	WMA
<b>CNS</b>	None	None	Migraine, MCI, polyneuropathy	Epilepsy	Epilepsy
<b>EMG</b>	Neurogenic	NA	Mixed signs	Myogenic	Myogenic
<b>Other features</b>	Asymmetric weakness	Myalgia	Distal legs involvement	Generalized muscle atrophy	Hyperlordosis, tiptoe gait

WMA: white matter abnormalities; MCI: mild cognitive impairment

**Table III.** Bioptical data of the patient described.

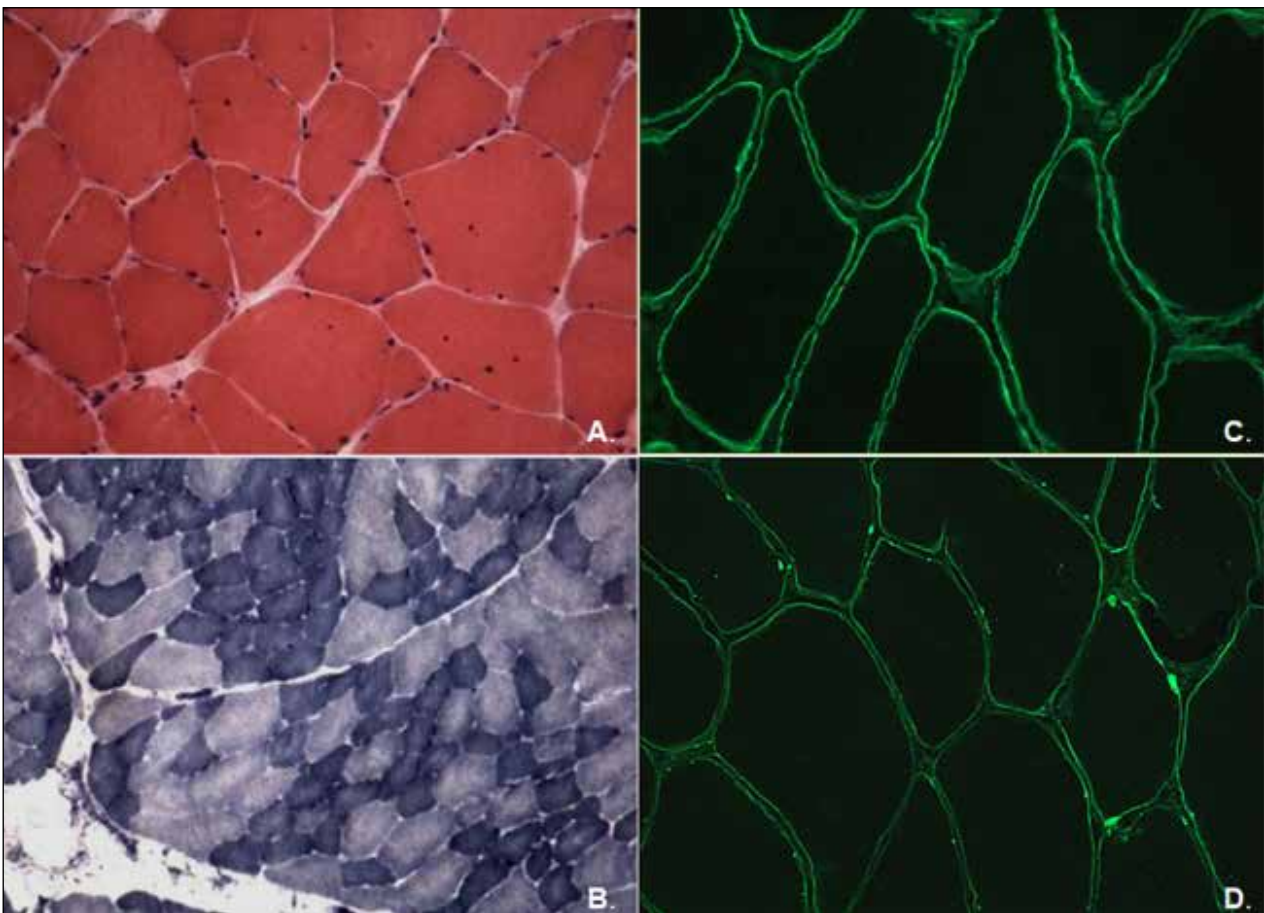
Patient	I	II.1	III	IV	V			
<b>Age at muscle biopsy (yrs)</b>	40	45	ND	67	75	ND	11	30
<b>Biopsy pattern</b>	Dystrophic	Myopathic	Dystrophic	Dystrophic	Dystrophic	ND	Myopathic	ND
<b>Merosin WB (residual %)</b>	ND	ND	30%	60%	60.00%	ND	ND	20%
<b>Merosin IHC 300 kDa</b>	Partial reduction	Partial reduction	ND	Normal/mild reduction	ND	ND	ND	Mild reduction
<b>Merosin IHC 80 kDa</b>	Partial reduction	Partial reduction	ND	Normal/mild reduction	Partial reduction	ND	ND	Mild reduction

imal atrophy (more pronounced at thigh and brachial biceps) and proximal lower limb weakness associated with bilateral calf hypertrophy. Weakness was asymmetric and involved predominantly the quadriceps (MRC 4+/5) and ileopsoas (MRC 4/5). At upper limb, brachial biceps was affected as well (MRC 3.5/5). Tendon reflexes were reduced at lower limbs. There was no distal, facial and bulbar involvement. Scoliosis, tendon retractions, cramps and myalgia were absent. The patient had a waddling hyperlordotic gate, possible on tiptoes but not on heels, and was able to walk for 400 meters at 6 minute-walking test. He raised from a chair pushing himself with upper limbs and was able to climb stairs with bilateral support. The patient lost the ability to raise from floor without external support at 55 years of age. Electrocardiogram and echocardiogram showed a normal cardiac function (ejection fraction 55%) and a right bundle branch block. Respiratory evaluation (spi-

rometry and nocturnal saturation) did not reveal pulmonary involvement. The patient did not show cognitive impairment.

The patient underwent two muscle biopsies respectively at the age of 40 and 45 years. The first biopsy, performed on quadriceps muscle, showed a dystrophic pattern with connective tissue substitution. The second biopsy, obtained from deltoid muscle, showed mild fiber size variability, nuclear centralization and fiber splittings, without necrosis or inflammation. Furthermore the connective tissue was normally represented (Fig. 1). At IHC analysis, dystrophin, sarcoglycans, alpha-dystroglycan, caveolin-3 and emerin showed a normal signal. WB study revealed a normal signal of dysferlin and alpha-dystroglycan and a mild reduction of calpain-3 without mutations in the corresponding gene.

At the age of 57 years, after two transient episodes of tinnitus and blurred vision, the patient underwent



**Figure 1.** Muscle biopsy Patient I. (A) Haematoxylin and eosin (H&E), 40x: few fibres with internal nuclei and fibre splittings; (B) Reduced nicotinamide adenine dinucleotide dehydrogenase- tetrazolium reductase (NADH-TR), 25x, showing mild variation in fibre size. Immunolabelling of laminin alfa2 to 80 kDa fragment in a control muscle (C) and in our case (D), showing a partial protein expression in some areas of the sarcolemma (arrows). (Immunolabelling with amino-terminus antibody is not shown).

brain MRI which showed radiological signs of leukoencephalopathy with bilateral, symmetric and diffuse T2 hyperintensity of peri- and sovra-ventricular white matter, extending to the subcortical regions and sparing U fibres. Posterior limb of internal capsule was only mildly involved. No signs of atrophy was found and the corpus callosum and posterior fossa structures were regular (Fig. 2). Eye examination was also normal.

According to the brain MRI finding, IHC and WB of merosin were performed. At IHC merosin labelling showed a partial reduction with both antibodies in few muscle fibers (Fig. 1), while the WB analysis showed a severe reduction of the protein expression (Fig. 3).

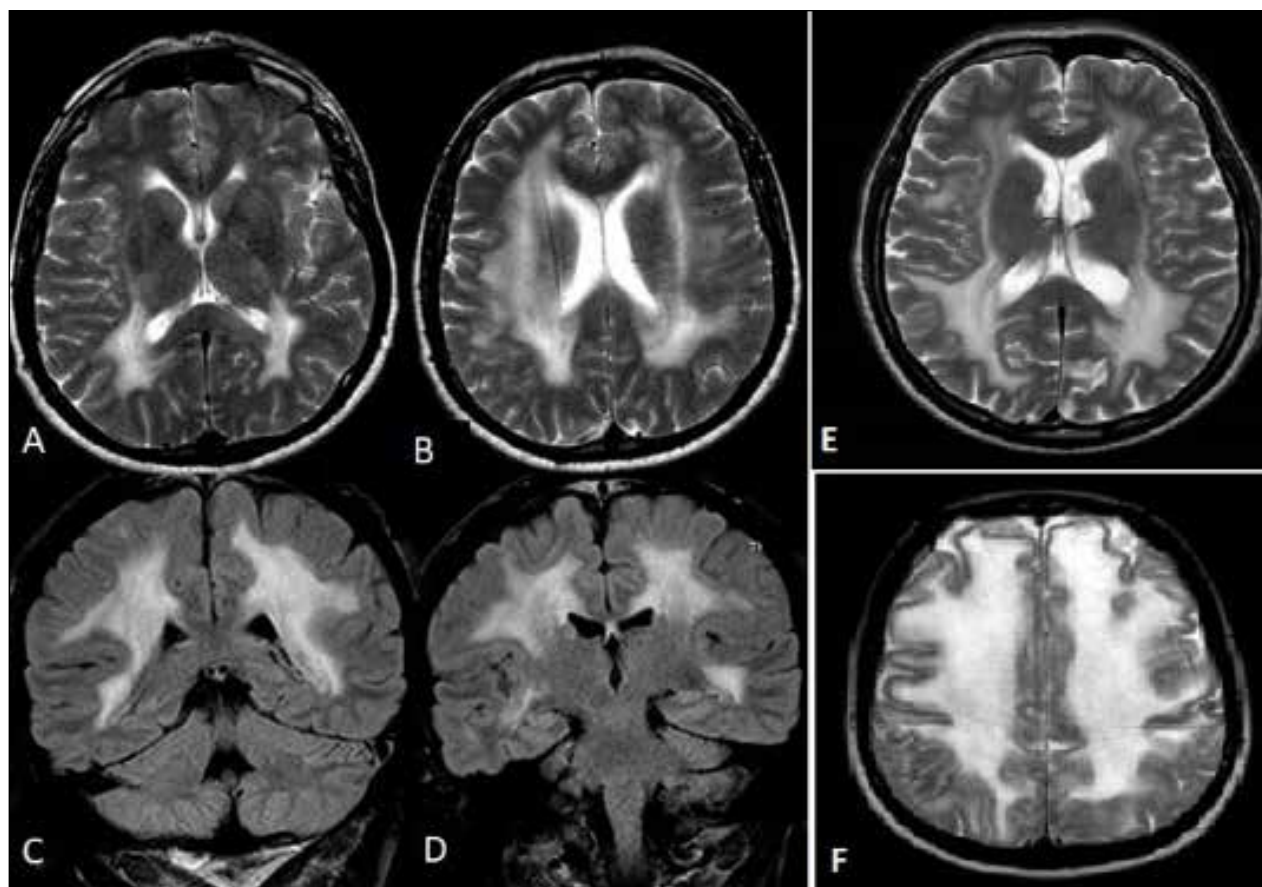
Muscle MRI, performed at 59 years of age, revealed a significant muscular impairment at both arms and thighs. In particular the most involved muscle of the upper limbs was the biceps brachii, followed by the triceps brachii, while deltoid was selectively spared. The muscles of the thigh showed atrophy and an high signal in T1-weighted images, which suggested fibro-fatty replacement. Some muscles were selectively spared, in particular the sarto-

rius, the rectus femoris and the short head of the biceps femoris. The right thigh was more impaired than the controlateral one, where also the semitendinosus muscle was partially spared. Atrophy and substitution were detected also in the medial portion of gastrocnemius. No inflammatory signs were found on STIR T2 images (Fig. 4).

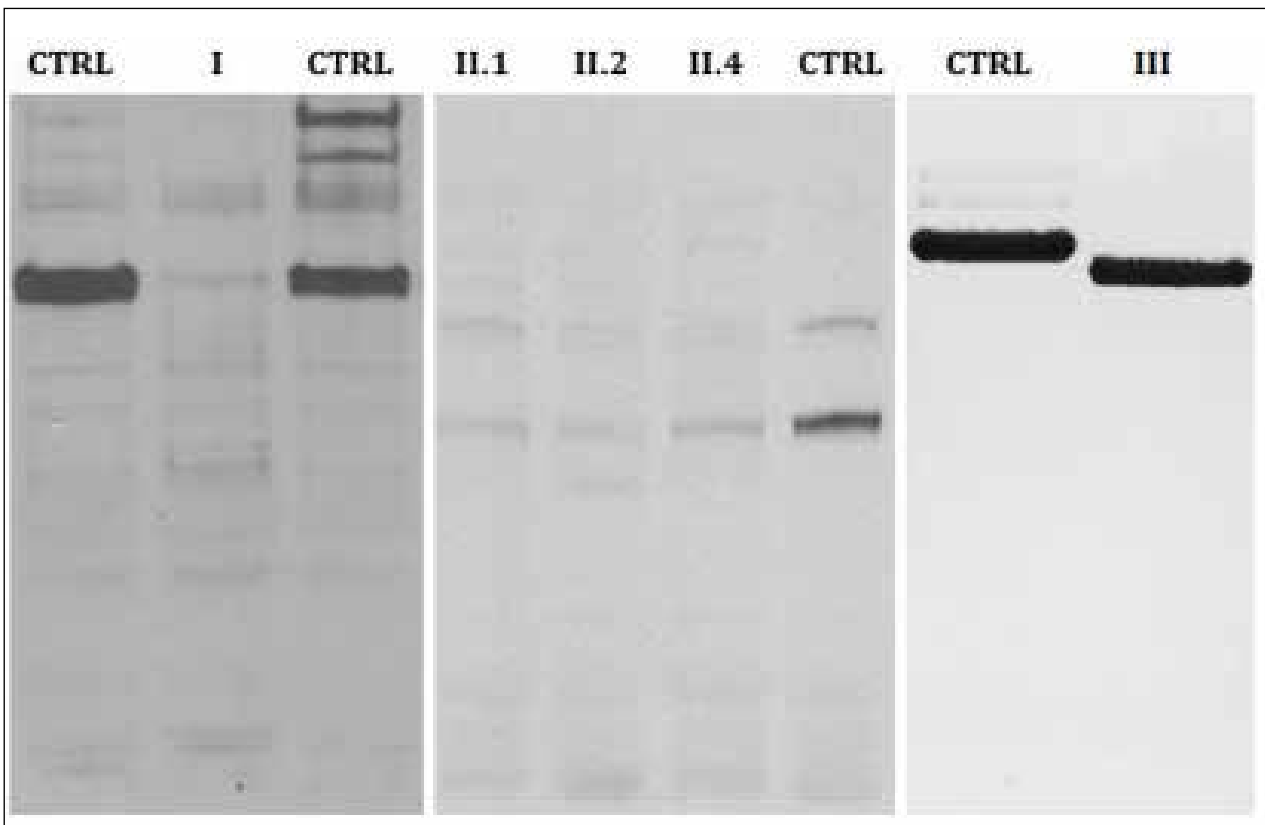
Molecular analysis showed the presence of two compound heterozygous mutation in *LAMA2* gene: the frame-shift c.6742delC (p.Leu2248TrpfsX23) in exon 48 and the missense mutation c.8544C > G (p.His2848Gln) in exon 60. Both mutations are novel. Both parents as far as the daughter of the patient carried the mutation in a heterozygous state.

#### Patient II

Subject II.1 is a 51-years-old woman presenting for progressive muscular weakness involving predominantly limb girdle muscles. She started to complain muscular weakness during childhood with difficulties in running. The disease showed a progressive course during the following years. The patient lost the ability of rising from



**Figure 2.** Brain MRI showing diffuse abnormal high signal intensity involving peri and sovra-ventricular white matter in axial T2 images (patient I: A-B; patient III: E; patient II: F) and coronal T2 FLAIR images (patient I: C-D).



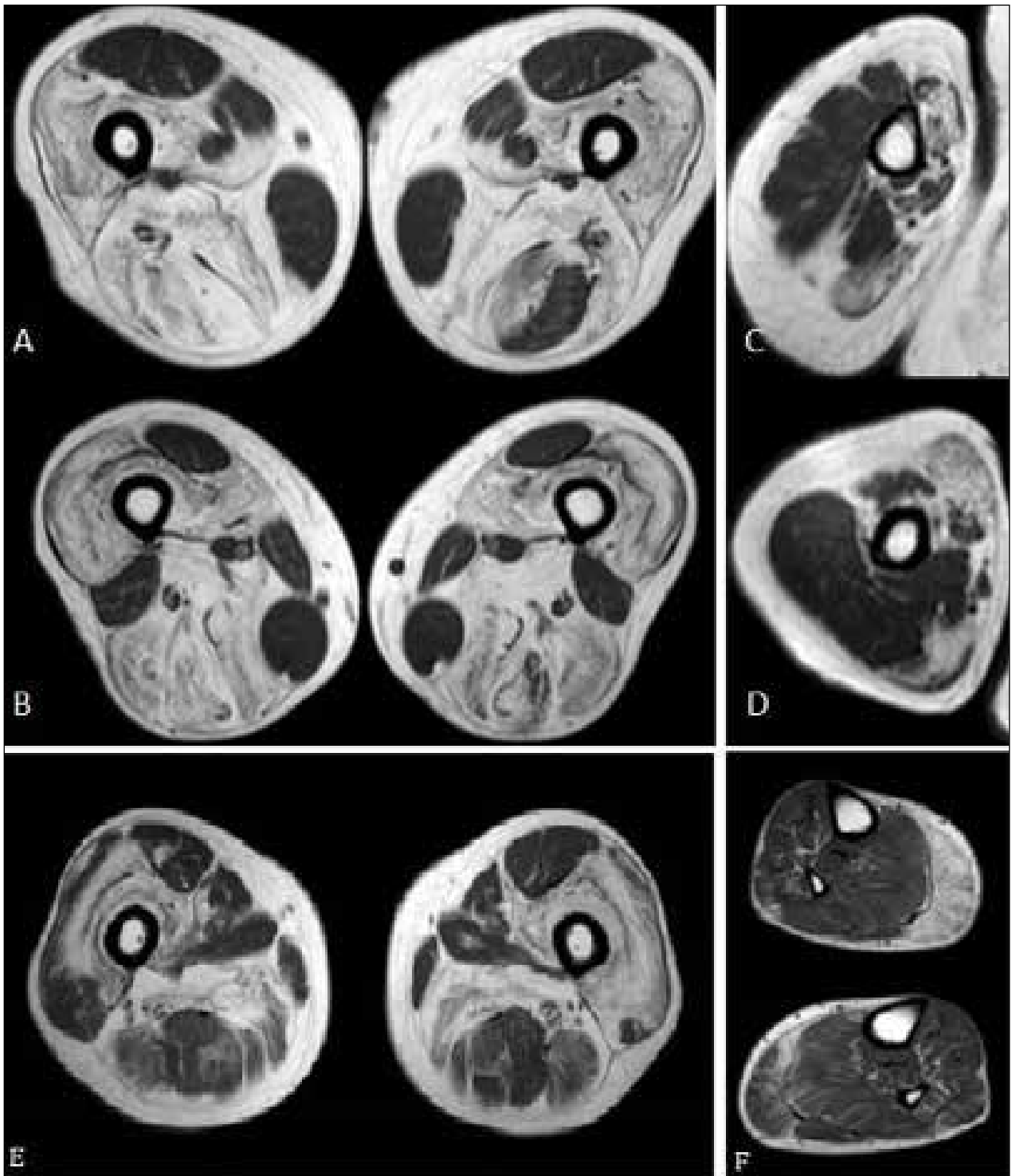
**Figure 3.** Western-blot analysis. Merosin Western-blot analysis showing a severe deficiency with monoclonal MAb 1922 antibody.

supine position at 10 years of age and of climbing stairs at 30 years of age. Myalgias were reported from adolescence. The neurological examination at 50 years of age showed symmetrical limb girdle weakness, sural pseudo-hypertrophy, bilateral mild Achilles tendon tightness, lumbar hyperlordosis and waddling gait. Weakness mainly involved lower limbs muscles, in particular gluteus (MRC 2/5), ileopsoas (MRC 4/5), quadriceps (MRC 4/5) and tibialis anterior (MRC 4/5). Deltoid (MRC 4/5) was the only muscle involved at upper limbs. The patient was able to raise from a chair only with external support. Cranial nerves and cognitive functions were normal. No episodes of loss of consciousness were reported. CK levels were mildly elevated (300-400 U/l). Pulmonary function was normal. The patient was affected by paroxysmal supraventricular tachycardia since the age of 45 years old and was treated with beta-blocker therapy.

Her parents were not apparently consanguineous but carried the same last name and were born in the same town. The patient had two unaffected children, but five relatives on her mother's side presented muscular involvement (Suppl. Fig. 1), suggesting a pseudo-dominant pattern of inheritance. The patient's mother (Subject II.2)

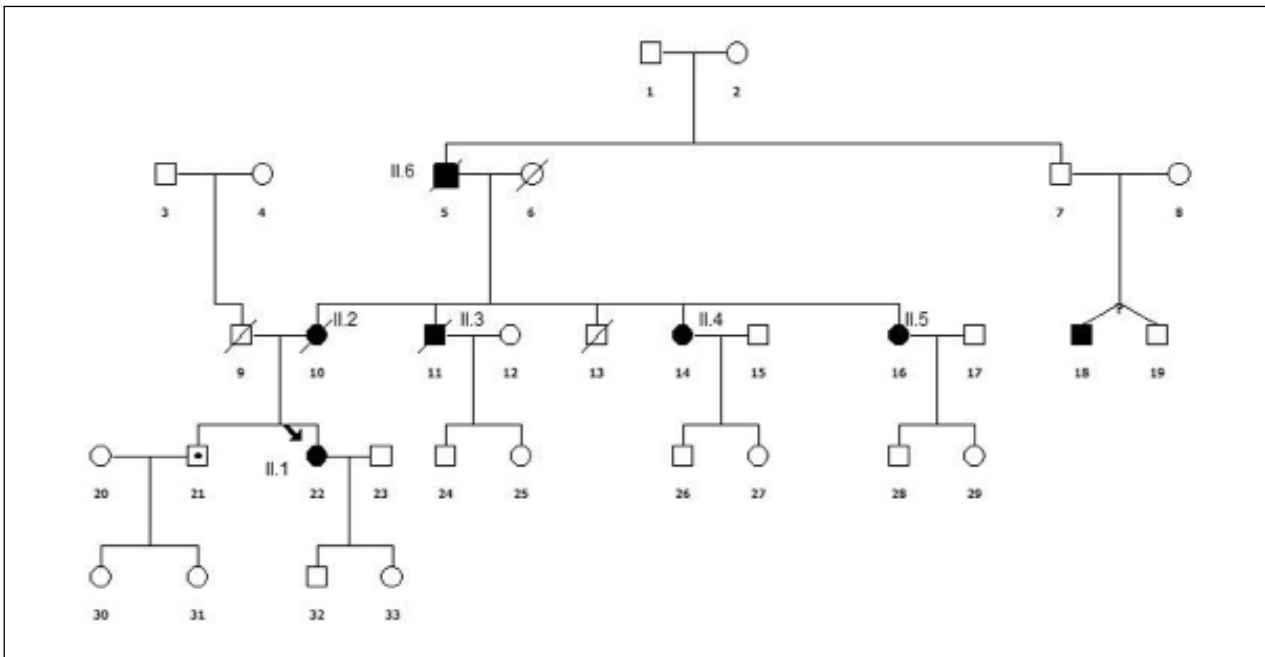
showed proximal muscular weakness and lost independent ambulation at 55 years of age. Three of her brothers and one sisters also showed neuromuscular involvement. In particular, one aunt (Subject II.5) lost ambulation at 60 years of age. Another uncle (Subject II.3) showed sural pseudohypertrophy since adolescence and lower limbs weakness since adulthood. In the following years he developed atrophy in gluteus and thigh muscles, waddling hyperlordotic gait and upper limb involvement with deltoid pseudo-hypertrophy and right fingers common extensor atrophy. His electromyography displayed myopathic signs. He died at 47 years of age in a road accident. Another aunt (Subject II.4) presented slowly progressive weakness in pelvic girdle muscles since childhood, associated with axial muscle involvement, sural pseudo-hypertrophy, lower limb areflexia, myopathic signs at EMG and CK increase (400 UI/L). At 71 years of age she required support for walking. Moreover, the maternal grandfather (Subject II.6) was also affected by an unspecified neuromuscular disease.

Muscle biopsy was performed in our proband and in subjects II.2 and II.4. The proband showed a dystrophic pattern, subject II.2 neurogenic alterations and subject



**Figure 4.** Muscle MRI. Axial TSE T1 images of the thighs (A-B) and of the right arm (C-D) of patient I, showing a diffuse atrophy and hyperintensity signal, as for fatty degeneration; some muscles were selectively spared, in particular the deltoid, the sartorius, the gracilis, the rectus femoris and the short head of the biceps femoris; the left thigh semitendinosus muscle was partially spared too. Axial T1 images of the thighs (E) and legs (F) of patient III, showing diffuse fibro-fatty substitution of the gluteal muscles, of the posterior thigh, adductor magnus and of the quadriceps; milder connective substitution in the medial gastrocnemius bilaterally.





**Suppl. Figure 1.** Pedigree of patient II suggesting a pseudo-dominant inheritance.

II.4 a myopathic pattern. In the proband immunohistochemical analysis for caveolin-3, alpha-sarcoglycan, gamma-sarcoglycan, alpha-dystroglycan, dysferlin and dystrophin were normal, while Calpain-3 (94 kD) was mildly reduced.

NGS analysis revealed an homozygous missense mutation (c.4405T > C; p.Lys1469Arg) in exon 30 of *LAMA2* gene. This mutation was previously described in compound heterozygosis in a patient with adulthood onset of mild proximal myopathy, polyneuropathy, dilated cardiomyopathy with conduction defects, histological features similar to Inclusion Body Myositis and partial laminin alpha-2 reduction<sup>17</sup>. This homozygous mutation was confirmed by Sanger sequencing both in the patient and in subject II.4. Unfortunately it was not possible to confirm the segregation in the other members of the family; however Western blot analysis was performed in subject II.1 II.2 and II.4 demonstrating severe merosin deficiency (30% of residual protein) in all of them (Fig. 3).

Brain MRI was performed in subject II.1 and revealed widespread periventricular and subcortical white matter hyperintensity in T2-weighted sequences and a reduction of ventricular system dimensions and cortical sulci, suggestive for white matter edema (Fig. 2). Signal abnormalities were found also in the corticospinal tract and to a lesser extent in the splenium of corpus callosum, posterior portion of the thalamus and dentate nucleus (basal ganglia were spared)<sup>25-28</sup>.

Muscle MRI, performed at 50 years of age, detected almost complete fatty substitution in thigh and leg muscles with relative sparing of the sartorius, gracilis, short head of the biceps femoral and tibialis posterior muscles. In the upper limbs the trapezius, supraspinatus, subscapularis, infraspinatus and pectoral muscles were mainly involved; milder signs of fibro-fatty infiltration were present also in deltoid muscles.

#### Patient III

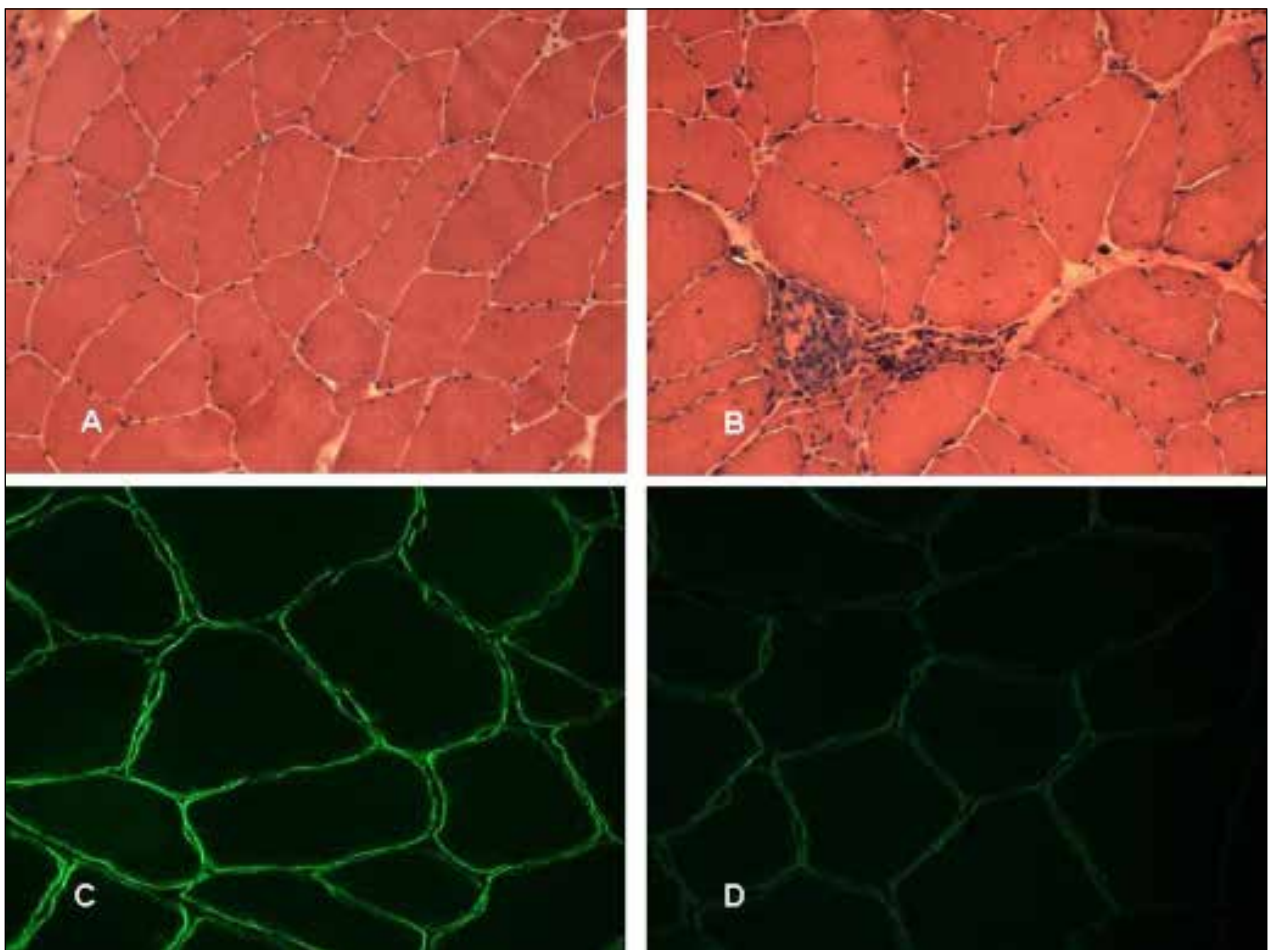
Patient III is a 75-year-old male, born from consanguineous parents (first cousins). He suffered from migraine since adolescence, almost unresponsive to common headache drugs. Family history was positive for dementia (the mother and two maternal aunts affected by). None of the other family members had neuromuscular disease history. The patient started complaining of mild exercise intolerance in his forties. Although he referred a worsening in fatigability, the muscular involvement remained almost stable over the years. At last evaluation he presents just a mild muscular weakness in distal legs associated with a mild calf hypertrophy. He always had high serum CK levels (500-800 U/l). The EMG showed a mixed neurogenic and mild myogenic pattern. EMG also documented a chronic polyneuropathy even if the patient has never complained neuropathic symptoms. Over the years the patient developed a dilated cardiomyopathy, recently worsened by atrial flutter episodes due to an abnormal ventricular pre-excitation, treated with catheter ablation when he was 73.

In his sixties a brain MRI, performed during the migraine follow-up, showed diffuse white matter lesions, spreading over the years to almost the whole brain white matter (Fig. 2), without clinical significant correlation. MMSE was of 26/30 and psychometric tests were globally normal. Because of headache, family history of dementia and MRI findings, a gene analysis for CADASIL was performed but turned out normal.

The patient underwent to muscle biopsies respectively at the age of 65 and 73 years (Fig. 5). The first biopsy showed some scattered COX negative fibres leading to a mitochondrial disease suspect. The immunofluorescence study for dystrophin, caveolin-3 resulted normal. Based on the suspicion of mitochondrial pathology, mtDNA long time PCR on muscle tissue was performed showing multiple deletions. Several nuclear genes involved in multiple deletions were screened

(POLG1, ANT1, POLG2, TYMP and PEO1) but turned out negative.

Eight years later, a second muscle biopsy and a muscle MRI were performed. The MRI showed a diffuse fibro-fatty substitution of the glutei, the posterior thigh, adductor magnus and quadriceps, sparing only the rectus femoris. A diffuse but milder connective substitution was also present in lower legs muscles, particularly in the medial gastrocnemius twins. The shoulder muscles were mild affected. No inflammation was detected (Fig. 4). The biopsy showed the presence of diffuse fibre atrophy and several necrotic fibres with a mild increase in muscular connective tissue. A few COX negative fibres were still present. At immunofluorescence, a mild, diffuse reduction of merosin alpha 2 came out (Fig. 5). Western Blot analysis of merosin on muscles shown a reduction of 40% in both the muscle biopsies (Fig. 3).



**Figure 5.** Muscle biopsy Patient III. Hematoxylin and Eosin stain on the the first muscle biopsy (A) and on the second (B). Scattered hypotrophic fibers in (A). Dystrophic pattern showing necrosis, nuclei centralizations and increase in connective tissue (B). Immunofluorescence staining (Merosin antibody) on the first muscle biopsy (C) and on the second (D). Original Magnification 40X (Merosin Laminin Alpha2). Normal Merosin binding (C) and scattered mild Merosin reduction of binding (D).

According to the MRI and bioptical results *LAMA2* gene was analyzed showing the presence of the following homozygous new mutation: c.2750+2 insT (IVS19), bringing to an in frame deletion of 75 nucleotides (c.2675-2749 del 75nt) as demonstrated by cDNA analysis. cDNA electrophoresis showed a band shorter than normal control, which corresponds with the production of a shorter protein product compared to the usual laminin size (p.Glu892Ala del893\_917). The healthy sister of the patient resulted heterozygotic carrier of the mutation.

#### Patient IV

Patient IV is a 42-year-old man with progressive muscular weakness starting during childhood. The parents described him as clumsy. He showed difficulties in running since 18 years of age and in climbing stairs since 30 years of age. He underwent surgery for tibio-tarsal retraction. He did not show cognitive involvement but presented since 35 years of age partial seizures. Cardiac and respiratory involvement were absent. Last neurological examination showed waddling gait, proximal weakness (2-4/5 at MRC evaluation) and elbow tendon retractions. CK levels were mildly increased (1640-677 U/L) and muscle biopsy showed mild connective tissue increase and fibre I atrophy. Merosin was not tested. Brain MRI showed widespread white matter alteration.

Molecular analysis of *LAMA2* gene revealed the presence of the following heterozygous mutations c.752T > C (p.Leu251Pro) in exon 5 and c.7586\_7589dup (p.Phe2531X) in exon 55, confirming the diagnosis.

#### Patient V

Patient V is a 53-year-old patient with negative family history. She came to medical attention at 30 years of age for progressive muscular weakness, with difficulties in climbing stairs and in rising from floor since 45 years of age. Retrospectively she presented motor delay with achievement of independent ambulation at 2 years of age and difficulties in running. At last evaluation neurological examination showed waddling gait, proximal weakness at four limbs (4/5 at MRC scale), tibio-tarsal retractions. CK were mildly elevated (180-680 U/L). Muscle biopsies showed myopathic signs. The patient did not show cognitive impairment. At 26 years of age she presented generalized seizures (both absences and tonic-clonic episodes). Brain MRI showed widespread alterations of brain white matter.

Molecular analysis showed the following compound heterozygous mutations in *LAMA2* gene: c.752T > C (p.Leu251Pro) in exon 5 and c.7147C > T (p.Arg2383X) in exon 50; both mutations were not previously described.

## Review of the literature

We performed a wide PubMed search of the literature concerning LGMD-like presentation associated to mutation in *LAMA2* gene. We used the most commonly used entries and selected only papers written in English and reporting a complete molecular characterization.

We selected 14 papers<sup>6-8,17-22,30-34</sup>. One paper<sup>30</sup> was excluded because it described three siblings with adulthood onset but predominant distal phenotype.

The clinical and molecular characteristics of the patients described in the remaining 13 papers are summarized in Table IV.

Overall 21 cases with LGMD presentation have been described since now. Age of onset spanned from 14 months to 59 years of age. However the patient with onset at 14 months complained waddling gait and frequent falls, but achieved independent ambulation at 12 months of age and did not show progression of weakness until 17 years of age. Only two patients showed mild delay in motor milestones, while all the other subjects achieved independent ambulation and had normal milestones. CK levels were moderately increased (average 1032 +/-926 UI/L, range 280-4100 UI/L). All patients were still ambulant at last evaluation (age range 11-65 years).

Contractures were reported in 6 patients, associated to rigid spine in two cases. One patient showed mild respiratory involvement while cardiac involvement was reported in two cases. Brain white matter abnormalities were present in all patients and were generally severe confluent white matter lesions, with exception of one case who showed only subtle bilateral signal abnormalities on the deep parietal lobe<sup>20</sup>. Cerebellar abnormalities were present in two subjects. Central nervous system involvement was variable and included epilepsy (3 patients), sporadic episodes of loss of consciousness (2 cases), mild mental retardation (2 subjects) and mild deficit of executive functions (2 patients). The remaining subjects had normal cognitive performances. Three patients showed peripheral neuropathy. Interestingly merosin staining generally showed partial reduction with exception of two cases which showed respectively absence at IHC study and severe reduction at WB analysis. In some cases merosin immunostaining revealed only a subtle decrease, in particular when antibody that recognizes the 80 kDa fragment was used, which could be erroneously interpreted as normal (Tab. III). Muscle biopsy showed a dystrophic pattern, rimmed vacuoles were present in three cases. As far as mutational analysis is concerned the majority of patients carried at least one missense mutation with the exception of the patient described by Di Blasi et al. which presented a nonsense mutation which however was associated with the skip-

**Table IV.** Review of the literature. LGMD patients with LAMA2 gene mutations described in literature: clinical and molecular characteristics.**Clinical aspects**

Pt	Sex	Onset (age)	Ambulation (age)	Brain MRI	CNS/PNS	CK	Other features	Ref
1	M	12 yrs	16 m	WMC	None	2417	Three sister affected	[16]
2	M	< 29 yrs	18 m	WMC	IQ 85	250-1236	Rimmed vacuoles; dilated cardiomyopathy, arrhythmias	[17,29]
3.1	M	15 yrs	Normal	WMC, cerebellar hypotrophy	Demyelinating neuropathy	4x	Severe contractures; mild respiratory involvement	[18,33]
3.2	F	Childhood	Hip dislocation	WMC	None	2x	Contractures	[18,33]
4	M	4 yrs	18 m	WMC	None	NA	Contractures	[32]
5	M	Childhood	18 m	WMC	Epilepsy, sensory-motor neuropathy	1429	Contractures	[31]
6	M	14 mo*	12 m	WMC	Low normal IQ, epilepsy	655	-	[6]
7.1	M	23 yrs	Normal	WMC	Epilepsy ?	309	-	[6]
7.2	F	40 yrs	2 yrs	WMC	Mild executive function deficit, epilepsy ?	405	-	[6]
8	M	10 yrs	Normal	WMC	Epilepsy	1053	-	[6]
9	M	59 yrs	Normal	WMC	Mild executive function deficit, trigeminal neuralgia	859	-	[6]
10.1	F	30 yrs	Normal	Subcortical and deep WMC	Epilepsy	280	Occasional rimmed vacuoles	[7]
10.2	M	NA	Normal	WMC	None	NA	Rimmed vacuoles	[7]
11	F	5 yrs	12 m	Deep parietal WMC	Sensorimotor neuropathy	653	-	[19]
12	M	56 yrs	Normal	WMC	None	1171	-	[8]
13	F	1 yr	Delayed	WMC	None	2148	Contractures	[8]
14	M	10 yrs	Normal	WMC	Epilepsy	1053	-	[8]
15.1	M	8 yrs	16 m	WMC	None	398-2103	Hyperreflexia	[21]
15.2	F	< 3 yrs	14 m	WMC	None	4100	-	[21]
16.1	M	Childhood	Delayed	WMC, globi pallidi involvement	None	400	Contractures, rigid spine, dilated cardiomyopathy, atrial fibrillation	[20]
16.2	M	7 yrs	Normal	ND	Sensorimotor neuropathy	400	Contracture, rigid spine	[20]

\* no progression until 17 yrs; WMC: white matter changes

**Table IV.** Review of the literature. LGMD patients with LAMA2 gene mutations described in literature: clinical and molecular characteristics.**Bioptical and molecular aspects**

Pt	Merosin ICH 300/80 kDa	Merosin WB	Molecular analysis	Ref
1	Severe / mild reduction	NA	Linkage analysis	[16]
2	Partial / partial reduction	Severe reduction	c.4405T > C (p.Cys1469Arg); c.4645C > T (p.Arg1549X)	[17,29]
3.1	Moderate / moderate reduction	Partial reduction	c.2230C > T (p.Arg744X + skipping of exon 15) in homozygosis	[18,33]
3.2	Slight / slight reduction	Partial reduction	c.2230C > T (p.Arg744X + skipping of exon 15) in homozygosis	[18,33]
4	Absent / absent	Absent	NA	[32]
5	Moderate / moderate reduction	NA	c.1847G > A (p.Gly600Arg) in homozygosis	[31]
6	Severe / mild reduction	50% reduction	c.850G > A homozygous (p.Gly284Arg)	[6]
7.1	Severe / mild reduction	65% reduction	c.728T > C (p.Leu243Pro); c.4860+2T > G_4860+3insGCC (p.Phe1573_Lys1620delinsSerfsX49)	[6]
7.2	ND	ND	c.728T > C (p.Leu243Pro); c.4860+2T > G_4860+3insGCC (p.Phe1573_Lys1620delinsSerfsX49)	[6]
8	ND / mild reduction	50% reduction	c.397-1_397-15del (p.Val133_Gln135delinsArgX5); c.7431A > T (p.Arg2477Ser)	[6]
9	NA	Absent	c.454T > G homozygous (p.Trp152Gly)	[6]
10.1	Mild reduction / normal	NA	c.2749+1G > A (Spl?); c.1177T > G (p.Cys393Gly)	[7]
10.2	NA	NA	c.2749+1G > A (Spl?); c.1177T > G (p.Cys393Gly)	[7]
11	Subtle / subtle reduction	Normal	c.391C > T (p.Gln131X); c.4487C > T (p.Ala496Val); (associated c.595T > A p.Cys199Ser exon 4)	[19]
12	Residual / NA	1/3 residual	NA	[8]
13	Residual /NA	Absent	c.3758T > GT (?) (p.Leu1253Arg); c.35T > G (p.Leu12Arg)	[8]
14	Residual / NA	1/3 residual	NA	[8]
15.1	NA / normal	NA	c.1358G > C (p.Cys453Ser); deletion exon 36-65	[21]
15.2	NA	NA	c.1358G > C (p.Cys453Ser); deletion exon 36-65	[21]
16.1	NA	NA	c.4533delT (p.Gly1512fsX); c.611C > T (p.Ser204Phe)	[20]
16.2	NA	NA	c.4533delT (p.Gly1512fsX); c.611C > T (p.Ser204Phe)	[20]

ping of the exon containing the mutation, thereby resulting in a restored open reading frame <sup>19</sup>.

## Discussion

Since now only 21 cases of patients with *LAMA2* gene mutations associated with adult-onset phenotype have been reported worldwide (Tab. IV). These cases are generally associated with partial reduction of merosin, the presence of almost one in-frame mutation, moderate increase of CK levels.

In this paper we describe 5 new subjects with *LAMA2* gene mutations associated with late onset phenotype characterized by mild, slowly evolving, proximal muscular involvement.

Mean age of onset was  $23.2 \pm 11.3$  years; all patients showed normal milestones and achievement of independent ambulation; only one patient was described as clumsy during childhood, all patients were ambulant at last evaluation. In particular, patients I and III showed a very mild clinical phenotype, with the latest age of onset (respectively 62 and 75 years of age) described since now.

Moreover patient III also showed a very mild muscular involvement. The other patients showed predominantly proximal muscle weakness, with distal involvement described only in one subject. CK were moderately elevated ( $640 \pm 267.8$  UI/L). Cardiac involvement was present in two patients who developed respectively paroxysmal supraventricular tachycardia and dilated cardiomyopathy associated to atrial flutter. Respiratory involvement was absent in all patients. Joint retraction and sural hypertrophy can be present. Electromyography showed a myopathic pattern occasionally associated to neurogenic signs (2 patients). Neurogenic signs were also detected in the biopsy of one patient (II.2). Peripheral neuropathy has been described in LAMA2-related diseases, mainly as a sensorimotor demyelinating polyneuropathy<sup>14,32</sup>, with possible axonal involvement<sup>20</sup> due to abnormal myelination linked to the deficient expression of merosin in the peripheral nerve Schwann cells. Two patients showed CNS involvement with epilepsy starting in adulthood, respectively at 26 and 35 years of age; seizures can be common in CMD but are rare in LGMD cases and generally start at younger age (Tab. IV). Migraine was present in patient III and never described before in association with this form of LGMD.

Brain MRI, which was abnormal in the majority of the patients, is fundamental to detect some specific aspects that can address the diagnosis. It should be performed even if central nervous system involvement is entirely subclinical. For example in patient I the molecular diagnosis was reached only after the accidental finding of white matter abnormalities at brain MRI, which addressed protein analysis and molecular investigation.

Muscle damage detected at muscle MRI is hard to compare with what is described in other patients because literature lacks MRI data in patients with partial merosin deficiency and LGMD phenotype. Only in a single report, a younger patient showed in her adolescence similar muscle MRI findings, although more severe than those found in the present series<sup>20</sup>.

Our patients showed diffuse atrophy and fatty degeneration of thighs with selective sparing of the sartorius, the gracilis, the rectus femoris and the short head of the biceps femoris also at later stages. A diffuse but milder connective substitution was also present at lower legs, particularly in the medial gastrocnemius twins while upper limbs were less affected. No inflammatory signs were found.

Molecular analysis showed seven different mutations in *LAMA2* gene, six of which are new. Merosin deficient patients with a LGMD phenotype carry at least one missense or in-frame mutation. Patient III carries the novel homozygous mutation IVS19+2 insT which affects splicing bringing to a 75 bp in-frame deletion in cDNA

transcript (c.2675-2749 del 75nt). We did not notice any correlation between mutations and disease severity.

Muscle biopsy analysis showed a myopathic pattern. In patient III the availability of two different muscle samples, collected at 13 years of distance, allowed to study disease progression from a histological point of view. In this case, at disease onset, disease epiphenomena like fibres atrophy and subtle mitochondrial abnormalities may lead to misdiagnosis.

All patients showed partial reduction of merosin at protein analysis. Western-blot data are available only in three patients, among them the patient with milder reduction (60% of residual protein) showed a later onset than the two patients with more severe deficiency (30% of residual protein), but this data should be confirmed in larger samples.

Furthermore we noticed a considerable variability in merosin IHC and WB staining pattern depending on the antibody used. This variability has been previously described. Jones et al. analysed with two different antibodies 58 muscle biopsy samples demonstrating that 40% of them showed a differential staining<sup>33</sup>. In particular the Chemicon antibody MAB1922, which was used in our patients, showed a milder degree of deficiency when compared to that observed with the Alexis MAB4H8-2. This significant difference between IHC and WB analysis results also by Di Blasi et al.<sup>18</sup>. Interestingly also Bushby et al.<sup>5</sup> described a group of patients with a late onset myopathy and the unusual finding of a reduction of merosin on immunoblotting but not on IHC analysis. The presence of severe merosin reduction at WB but not at IHC in this group of patients and in our probands could be explained by different reactivity of the antibody, by the extensive processing of homogenization which is necessary for WB analysis or by a patient's specific predisposition to merosin fragility.

Furthermore in some cases protein analysis could be misleading because, especially when only amino-terminus antibodies are used, it can underestimate merosin deficiency. WB analysis with antibodies directed against both the amino- and carboxyl-terminus of the protein must therefore be used when a LGMD associated to merosin deficiency is suspected.

## Conclusions

Since now only few cases of LGMD with *LAMA2* gene mutations have been described. LGMD R23 is a rare cause of LGMD, although this entity might be under-recognized. This could obviously be due to its rarity, but also to the fact that some contributory examinations, such as brain MRI, are not routinely performed in LGMD patients.

Overall the description of our cases further enlarges the spectrum of clinical phenotypes associated to mutations in *LAMA2* gene. In particular our case sample includes patients with very late age of onset and very mild muscular involvement. Furthermore, the description of muscle MRI in our sample expands the knowledge about muscle imaging in this form of LGMD. WB analysis and brain MRI should be useful in order to suspect this form of LGMD also in pauci-symptomatic patients.

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