Frequency and characterisation of anoctamin 5 mutations in a cohort of Italian limb-girdle muscular dystrophy patients

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Abstract

Limb-girdle muscular dystrophy (LGMD) 2L, caused by mutations in the anoctamin 5 (ANO5) gene, is the third most common LGMD in Northern and Central Europe, where the c.191dupA mutation causes the majority of cases. We evaluated data from 228 Italian LGMD patients to determine the prevalence of LGMD2L and the c.191dupA mutation, and to describe the clinical, muscle biopsy, and magnetic resonance imaging findings in these patients. Forty-three patients who lacked molecular diagnosis were studied for ANO5 mutations, and four novel mutations were found in three probands. Only one proband carried the c.191dupA mutation, which was compound heterozygous with c.2516T>G. Two probands were homozygous for the c.1627dupA and c.397A>T mutations, respectively, while a fourth proband had a compound heterozygous status (c.220C>T and c.1609T>C). Therefore occurrence and molecular epidemiology of LGMD2L in this Italian cohort differed from those observed in other European countries. ANO5 mutations accounted for ~2% of our sample. Affected patients exhibited benign progression with variable onset and an absence of cardiac and respiratory impairment; muscle biopsy generally showed mild signs, except when performed on the quadriceps muscles; MRI showed predominant involvement of the posterior thigh. Overall these common clinical, morphological and imaging findings could be useful in differential diagnosis.

Keywords: Limb girdle muscular dystrophy 2L; Quadriceps myopathy; Anoctamin 5; Chloride channel; Membrane repair

1. Introduction

Limb-girdle muscular dystrophy (LGMD) 2L is a recessive form of LGMD described for the first time in 2010 [1].

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The disease is caused by mutations in the anoctamin 5 (ANO5) gene, which contains 22 exons and maps to chromosome 11. Recessive mutations in this gene have also been reported to cause a distal non-dysferlin Miyoshi-like myopathy (MM3) [2], whereas dominant variations can determine gnathodiaphyseal dysplasia (GDD) [3]. Anoctamin 5 is a member of the anoctamin family, which comprises at least ten proteins, each consisting of eight transmembrane domains and a DUF590 domain of
unknown function [4]. These proteins are widely expressed and reportedly act as calcium-activated chloride channels. In particular, anoctamin 5 is expressed in skeletal muscle and the heart, where it is localised in intracellular vesicles and the endoplasmic reticulum [5] and seems to participate in membrane repair [6].

The existence of a novel autosomal recessive LGMD was suggested in 2007 by Jarry et al. [7]; in a cohort of French Canadian families, they described a form with prominent asymmetrical atrophy of the quadriceps femoris and biceps brachii that seemed to map to chromosome 11p13-p12. Three years later, a study with a larger sample of patients better defined the involved region as 11p14.3-p15, leading to the identification of ANO5 as the responsible gene [1]. Thus far, other cases have been described in populations from Northern England, Finland, Germany and France [2,8–12]. This form seems to be the third most common form of LGMD in Northern England [8] behind LGMD2A and LGMD2I. Overall, 49 families and 19 different mutations have been described in the literature, without hot-spots along the gene although the 60% of mutations occur in the 5’ portion of the gene (between exon 15 and 20). The c.191dupA mutation is responsible for the majority of cases; it was described as homozygous in 14 families and as compound heterozygous with another mutation in 16 families, suggesting a founder effect in Northern Europe populations [8]. The c.2272C>T is particularly frequent in Finnish population, since it was found in 20 independent families. Among the other 17 mutations that were detected, the c.1407+5G>A substitution was reported three times, the c.1295C>G, c.172C>T, c.2018A>G, c.692G>T mutations twice, and the remaining mutations only once (Fig. 1A). All patients show some common clinical aspects, namely predominant lower limb involvement with impairment of the posterior compartment muscles, mild to significant increase in creatine kinase (CK), adulthood onset, slow progression, and absence of cardiac and respiratory involvement [8].

However, all of these data are based on the analysis of a small sample of patients limited to Northern Europe. Further studies are needed to analyse the prevalence of this muscular dystrophy in other countries and to better define both the clinical presentation and prognostic factors. Here, we report a study of an Italian sample of 228 LGMD patients in order to establish the prevalence of LGMD2L in Italy and to better define the clinical, morphological, and molecular features of this form.

2. Materials and methods

2.1. Patient selection

We selected 43 LGMD patients (37 families) without a molecular diagnosis from a cohort of 228 LGMD patients from 190 families that was previously examined at our neuromuscular clinic. The 153 remaining probands had a molecularly proven diagnosis of LGMD, distributed as follows: 59 LGMD2A, 42 LGMD2B, 9 LGMD2C, 16 LGMD2D, 8 LGMD2E, 1 LGMD2F, 14 LGMD2I, and 3 LGMD1C. Patients were defined as affected with LGMD if they fulfilled the following criteria: a clinical phenotype characterised by progressive muscle weakness and wasting affecting primarily the shoulder girdle and pelvic muscles, keeping with the diagnostic criteria for LGMD [13], and dystrophic features at muscle biopsy. Patients had all undergone a systematic clinical characterisation, including comprehensive neurological, cardiac (electrocardiogram and echocardiogram), and respiratory (spirometry) assessments. Muscle imaging data were also obtained by nuclear magnetic resonance imaging (MRI) or computed

![Fig. 1. Schematic representation of the intron/exon organisation of ANO5 and distribution of mutations along the gene. (A) Mutations already described in the literature. In the box, the mutations that are reported more than once are highlighted. (B) Mutations found in our cohort. Homozygous or compound heterozygous mutations in blue, and heterozygous mutations in green.](image-url)
tomography (CT) when available. All specimens were obtained from the “DNA, Muscle and Nerve Tissue Bank” of the Neurological Unit, Fondazione IRCCS Ca’ Granda Ospedale Maggiore Policlinico, University of Milan.

Written informed consent was obtained from all subjects or their caregivers when primary diagnostic procedures were performed, with explicit consent for future use for research purposes, according to the Declaration of Helsinki. This protocol was approved by the Institutional Review Board. Overall, 90% of patients were from Italian families; the remaining subjects were from other countries (Switzerland, Tunisia, and Albania). These latter patients were not considered in the evaluation of the population-based proportion of LGMDs.

2.2. Muscle tissue analysis

All probands underwent muscle biopsy of the brachial biceps or quadriceps femorii muscles. Morphological examination was performed following standardised methods. We studied the most important proteins involved in LGMDs using immunohistochemical (IHC) analyses with monoclonal antibodies directed against dystrophin (Novocastra, Newcastle upon Tyne, UK) [14], sarcoglycans, dysferlin (Novocastra), α-dystroglycan (α-DG) (Upstate Biotechnology, Lake Placid, NY), and caveolin-3 (Transduction Laboratories, Lexington, KY) [15]. The protein defects uncovered by IHC analysis were confirmed by Western blotting using the same monoclonal antibodies. This technique was also used for the calpain-3 screening using the Novocastra monoclonal antibody Calp 3d/2C4 [17]. For electron microscopy (EM) studies, muscle samples were fixed in glutaraldehyde and processed using standard procedures [18].

2.3. Molecular analysis

Genomic DNA was extracted from peripheral blood samples according to standard procedures (Flexi Gene DNA Handbook, Qiagen). All patients were screened for the c.191dupA mutation, which is commonly found in English and German populations, by direct sequencing of the PCR products using an ABI PRISM 3100 XL Genetic Analyzer (Applied Biosystems). In patients without mutations, we used the same method to analyse all 22 ANO5 exons and their flanking intronic regions. Primer sequences and PCR conditions are available upon request.

The pathogenic nature of new mutations was confirmed by screening 160 healthy control subjects, and the parental origin of each mutation was assessed from the parental genomic DNA when available. Amino acid conservation was confirmed by comparing sequences in different species. For some patients, mRNA was isolated from muscle tissue using Eurozol. 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.

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.

3. Results

We studied ANO5 in 43 patients (37 families) affected with LGMD but lacking a molecular diagnosis. Homozygous or compound heterozygous causative mutations were found in four probands (six patients). Only one patient carried the heterozygous c.191dupA mutation that was common in previous studies. Four novel mutations were detected (Fig. 1).

The molecular and clinical features of the patients are summarised in Tables 1 and 2.

Two probands (three patients) carried single heterozygous mutations. They were characterised by more heterogeneous clinical presentations.

3.1. Patient I

Patient I was a 47-year-old man delivered after a regular pregnancy, with normal psychomotor development and negative family history (Fig. 2A). During childhood and adulthood the patient played soccer at a competitive level. In early adulthood, altered liver enzyme values had been found but never investigated. At 31 years of age the patient experienced an episode of myoglobinuria with high CK levels (10,000 UI/L). Since that time, the disease showed a progressive course with onset of mild weakness of the lower limbs starting at 36 years of age, and difficulties climbing stairs and rising from a chair at 40 years of age. CK levels were moderately increased (6- to 11-fold above normal). Electromyography showed myopathic changes.

The patient underwent two muscle biopsies. The first biopsy, performed at 31 years of age on the quadriceps femorii, revealed a severe dystrophic pattern with nuclear centralisation and increased connective tissue. The second biopsy, performed at the age of 46 years on the brachial biceps, showed a milder pattern with some central nuclei and necrotic fibres, but no increase in connective tissue. Dystrophin immunostaining and Western blot results were normal. We found a mild reduction in the 30-kD calpain-3 band, but molecular analysis of calpain-3 did not reveal any mutations. An ultrastructural study demonstrated basal lamina duplication (data not shown) and focal loss of the plasma membrane with sporadic thickening of the basal lamina overlying the plasma membrane defect (Fig. 3C and D).

Neurological examination showed a predominant lower limb involvement, including severe asymmetric muscle atrophy of the quadriceps and posterior compartment of the leg (Fig. 4A) with selective quadriceps and hamstring weakness. Upper limb muscle bulk and strength were normal. Cardiac and respiratory involvement was absent.
Interestingly, during the disease course, this patient was subjected to several muscle imaging studies, both with MRI and CT scan. These studies clearly underlined the progression of the disease. The first CT scan, performed at 29 years of age, revealed muscle atrophy and increased signal intensity on T2-weighted images, consistent with muscle dystrophy. Over the subsequent years, serial imaging showed progressive atrophy and increased muscle mass, indicating the relentless nature of the disease.

### Table 1
ANO5 gene mutations in our cohort.

| Allele 1 | Allele 2 |
|----------|----------|
| Pt | Nucleotide | Protein | Exon | Reference | Nucleotide | Protein | Exon | Reference |
| I | c.220C>T | p.Arg74X | 5 | New | c.1609T>C | p.Ser537Pro | 15 | New |
| II.1 | c.1627dupA | p.Met543AsnfsX10 | 15 | Penttila (2012) | c.1627dupA | p.Met543AsnfsX10 | 15 | Penttila (2012) |
| II.2 | c.1627dupA | p.Met543AsnfsX10 | 15 | Penttila (2012) | c.1627dupA | p.Met543AsnfsX10 | 15 | Penttila (2012) |
| III.1 | c.191dupA | p.Asn64LysfsX15 | 5 | Brais (2010) | c.2516T>G | p.Met839Arg | 21 | New |
| III.2 | c.191dupA | p.Asn64LysfsX15 | 5 | Brais (2010) | c.2516T>G | p.Met839Arg | 21 | New |
| IV | c.397A>T | p.Ile133Phe | 7 | New | c.397A>T | p.Ile133Phe | 7 | New |

### Table 2
Clinical characteristics of LGMD2L patients.

| Pt | Age (yrs) | Gender | Onset (yrs) | Clinical features | Symptoms at onset | Atrophy distribution | Muscle weakness | CH | C | My | Mg | Cardiac involvement | Respiratory involvement | EMG | CPK | Clinical evolution |
|----|-----------|--------|------------|------------------|------------------|---------------------|------------------|----|---|----|----|-------------------|----------------------|-----|-----|-------------------|
| I  | 47        | M      | 39         | LGMD (lower limbs) | Myoglobinuria     | Quadriceps, posterior compartment, Harmstrings, quadriceps | Quadriceps and biceps femori | –  | – | +  | –  | None              | None                  | Myopathic | 6-11x | Slow             |
| II.1 | 51        | M      | 15         | LGMD + distal involvement | Mild weakness | Quadriceps, biceps femori | Quadriceps, biceps femori | +  | + | +  | –  | None              | None                  | Myopathic | 5-32x | Slow             |
| II.2 | 43        | M      | 33         | LGMD (almost asymptomatic, mainly lower limbs) | Asymptomatic hyperCKemia | Biceps femori | Quadriceps, posterior compartment, Harmstrings, quadriceps | Quadriceps | ++ | –  | –  | +  | None              | None                  | Myopathic | 15-31x | Slow             |
| III.1 | 45        | M      | 28         | LGMD (lower limbs) | Fatigability and cramps | Quadriceps | Quadriceps | +  | +  | +  | Nd | None              | None                  | Myopathic | 16x   | Slow             |
| III.2 | 47        | F      | Nd         | HyperCKemia | None | None | None | –  | –  | –  | –  | None              | None                  | Not performed | 3x   | Nd               |
| IV  | 50        | M      | 47         | LGMD (four limbs) | Myalgias and hypostenia | Asymmetric quadriceps | Quadriceps | –  | +  | +  | +  | None              | None                  | Myopathic | 2-7x  | Slow             |

CH: calf pseudohypertrophy, C: cramps; My: myalgia; Mg: myoglobinuria.

Fig. 2. Pedigrees of the mutated families. Familiars that were tested for ANO5 mutations are specified in each pedigree. WT: wild-type.
of age, showed only atrophy of biceps femoris, but progressive involvement of the gastrocnemius and soleus muscles became evident over the years (latest study at 43 years of age). The sartorius, semitendinous muscles, and the anterior compartment of the leg were preserved (Fig. 5), with exception of the quadriceps muscles, which were involved with a patchy pattern. The involved muscles showed mild hypodensity at the beginning of the illness (Fig. 5A and B) with progressive substitution of fatty tissue occurring over the years (Fig. 5C and D).

Molecular analysis of ANOS5 revealed the presence of two heterozygous mutations (Fig. 6A). The first mutation (c.220C>T; p.Arg74X in exon 5) was a nonsense mutation located in the N-terminal intracytoplasmic loop of the protein. The second mutation (c.1609T>C; p.Ser537Pro in exon 15) was a missense mutation in a highly conserved
portion of the gene, affecting the loop between transmembrane domains 4 and 5 (Fig. 6E).

3.2. Patients II.1 and II.2

Patients II.1 and II.2 were brothers born from consanguineous parents. They had three siblings without muscular impairment (Fig. 2B). The older brother (patient II.1) was a 51-year-old man with normal physical development; in childhood and early adulthood he had participated in martial arts at a competitive level. At 36 years of age, patient II.1 was diagnosed with HIV and started antiretroviral therapy without relevant side effects. At 39 years old, the patient complained of weakness in his lower limbs with difficulty in walking for long stretches. He underwent a muscle biopsy of the brachial biceps, which showed mild dystrophic and inflammatory signs, and was treated for 7 years with steroid therapy with only mild benefit. CK levels were consistently elevated at onset (32-fold) and mildly increased in the following evaluations (5-fold).

Patient II.2 was a 43-year-old man who sought medical attention at 31 years of age due to an incidental finding of asymptomatic hyperCKemia (7000 UI/L). Two years later, the patient started to complain of mild proximal weakness in all four limbs, though he currently works as a tire repairer. The patient experienced one episode of myoglobinuria. At the age of 31 years, patient II.2 underwent muscle biopsy of the biceps brachii, which showed a mild increase in connective tissue, nuclear centralisation, and fibre splitting (Fig. 3A).

Molecular analysis showed that both brothers had homozygous single nucleotide duplication (c.1627dupA) in exon 15 that disrupted the transcript reading frame (p.Met543AsnfsX10) (Fig. 6B). Similar to the mutation in patient I, this mutation is located in the intracytoplasmic loop between transmembrane domains 4 and 5 (Fig. 6E).

Interestingly, neurological examination showed that both brothers (Fig. 4B and C) had asymmetrical calf pseudohypertrophy, though it was more marked in patient II.2, and hamstring atrophy and weakness (score of 3 on the Medical Research Council (MRC) scale in patient II.1 and 4 in patient II.2). Patient II.1 also showed quadriceps weakness (score of 3 on the MRC scale) and mild upper girdle involvement (4 on the MRC scale). Both brothers had normal distal strength, as they were able to walk on both their toes and heels, and did not have cardiac or respiratory involvement. Muscle MRI performed in patient II.2 when he was 42 years old showed bilateral adipose substitution of the posterior compartment of the thigh with partial atrophy of the quadriceps and adipose substitution of the soleus muscle (Fig. 5B).

3.3. Patients III.1 and III.2

Patient III.1 was a 45-year-old man who presented at 28 years of age with fatigability and cramps. Over the following years, the patient developed pelvic girdle weakness. He underwent three muscle biopsies, at 28, 41, and 42 years of age; all of the biopsies showed myopathic changes with increased connective tissue, nuclear centralisation, and

Fig. 5. Muscle imaging. (A–D) Muscle CT imaging of patient I at 29 years of age (A and B) and 43 years of age (C and D). The first study demonstrated only mild atrophy of the biceps femoris, whereas during the following years progressive involvement of the quadriceps femoris, gastrocnemius, and soleus muscles was observed. (E and F) MRI of the muscle of patient II.2, demonstrating bilateral adipose substitution of the posterior compartment of the thigh with partial atrophy of the quadriceps.
fibre splitting. In two samples, both IHC and Western blot analysis showed altered dystrophin expression, especially with antibodies directed at the N-terminal domain. Molecular analysis of dystrophin with Multiplex-PCR and MLPA did not reveal any mutations. Muscle CT at 43 years of age demonstrated adipose infiltration, mainly in the posterior thigh compartment and quadriceps.

At the neurological examination, the patient presented calf pseudohypertrophy, quadriceps atrophy, and proximal weakness. Cardiac and respiratory evaluations showed a normal pattern. ANO5 sequencing revealed the presence of the common c.191dupA mutation, as well as the novel missense substitution in exon 21, c.2516T>G p.Met839Arg (Fig. 6C) in the transmembrane domain (Fig. 6E).

Molecular analysis was extended to the other members of the pedigree. The patient had three sisters (Fig. 2C), one of whom (patient III.2) carried the same compound heterozygous mutations and had mild hyperCKemia (3-fold) without abnormalities at neurological examination and with no referred weakness. Among the other two sisters, one carried the heterozygous c.191dupA mutation, and the other had a wild-type genotype. The mother harboured the heterozygous c.2516T>G substitution.

3.4. Patient IV

Patient IV was a 50-year-old man born from consanguineous Tunisian parents (Fig. 2D). His family history was negative for neuromuscular disorders, and the patient’s first symptoms started at 47 years of age when he began to complain of mild proximal weakness with difficulties lifting weights, cramps, and severe myalgia. The patient also reported some episodes of myoglobinuria, his CK levels were mildly increased (2- to 8-fold above normal values).

At the age of 47 years, the patient underwent muscle biopsy of the brachial biceps, which showed mild myopathic changes with several nuclear centralisation, few split fibres, and no connective tissue increase (Fig. 3B). Ultrastructural analysis demonstrated the presence of basal lamina duplication and separation from the underlying sarcoplasm (Fig. 3E and F).

Molecular analysis of ANO5 revealed the presence of a previously undescribed homozygous missense mutation in exon 7, c.397A>T (p.Ile133Phe) (Fig. 6D). This mutation is localised in the N-terminal loop of the protein in a portion of the gene that is well preserved among species (Fig. 6E). The patient’s sister and brother carried the heterozygous c.397A>T mutation and were completely asymptomatic.

Neuromuscular examination showed predominant lower limb involvement with severe asymmetric atrophy of both the quadriceps and the muscles of the posterior compartment (Fig. 4D). Strength was normal except for the quadriceps, which had a score of 4 on the MRC scale. The patient also had mild muscle weakness of the shoulder girdle without atrophy. Distal strength was normal, and the patient was able to walk on his toes and heels. Tendon reflexes were absent. Overall, the motor impairment was moderate. The patient did not have cardiac or respiratory involvement.

3.5. Patients V.1, V.2, and VI

Patients with heterozygous mutations of ANO5 were characterised by more heterogeneous clinical presentation. Patients V.1 and V.2 each had a precocious onset, at 3 and 6 years of age, respectively. At their present ages of 46 and 56 years, both patients presented with weakness at the shoulder girdle and distally in their lower legs. CK levels are only mildly increased (2-fold) and both patients presented a respiratory restrictive pattern. Muscle biopsy of patient V.1 revealed severe dystrophic involvement with marked connective tissue increase.

Genetic analysis revealed the missense mutation c.G892A, p.Glu298Lys, localised near the first transmembrane domain of the protein in a region that is well conserved among species. Neither ANO5 genomic sequencing, nor mRNA analysis demonstrated the presence of a second mutation.

Patient VI was a 21-year-old boy who presented at 6 years of age with cramps and weakness. He did not have cardiac or respiratory involvement. He currently shows mild limb girdle weakness with tendon retraction. The patient’s CK levels fluctuated from normal values up to 12-fold the normal values, and muscle biopsy showed a severe dystrophic pattern. Sequencing of ANO5 revealed a missense substitution, c.T1733C (p.Phe578Ser). This mutation was previously described once in the literature in association with the common mutation in a patient presenting with late-onset LGMD and mild progression [8]. The father and sister of the patient carried the same mutation and were completely asymptomatic, so we excluded the possibility that this ANO5 sequence variant acts as a dominant mutation. Unfortunately, we did not have a muscle sample or blood sample from this patient to analyse cDNA and search for a second mutation.

4. Discussion

4.1. Incidence of ANO5 mutations and molecular analysis

ANO5 screening of 43 patients (37 families) affected with LGMD without molecular diagnosis revealed homozygous or compound heterozygous mutations in six patients belonging to four independent families. Three patients (two probands) carried single heterozygous mutations but were not included in the analysis. These patients exhibit some peculiar clinical aspects and should probably be considered carriers of ANO5 mutations. Reporting their genetic variants may help build a reference database on the genetic variation of ANO5.

Overall, mutations in ANO5 seem to be responsible for 2% (4/157) of LGMDs diagnosed in our Italian sample.
This incidence is lower than that of Northern European countries, where LGMD2L is considered the third most common form of LGMD, following LGMD2A and LGMD2I [8]. In Italy, LGMD is still most commonly due to mutations in the genes encoding calpain-3, dysferlin, sarcoglycans, and fukutin-related protein (FKRP).

Among homozygous or compound heterozygous patients, we found six different mutations in \( \text{ANO5} \), four of which are novel (Fig. 1). The common \( \text{c.191dupA} \) mutation was substantially less frequent than previously described in the literature and was found in only one patient, compound heterozygous with a missense mutation. Conversely, almost all patients from Northern England and Germany carry the \( \text{c.191dupA} \) mutation as a homozygous or compound heterozygous mutation, suggesting a founder effect. None of our patients carry the \( \text{c.2272C>T} \) mutation, which is common in Finnish population.

These data suggest a different geographical distribution of \( \text{c.191dupA} \) and \( \text{c.2272C>T} \) mutations. A similar different distribution has been observed for the frequency of LGMD2I [19]; the estimated frequency of FKRP-mutated LGMD in North-European populations ranges from 16% to 38%, [20,21] while in Italy figures of 5% were reported [19,22]. However, the common FKRP mutation has been found in Italian and North-European LGMD2I patients [19–21], while this does not seem to be the case for LGMD.
2L, making it impossible to use the screening of common mutation(s) as a first approach in the molecular study of LGMD2L in our population.

Overall, half of our mutations (3/6) were missense. A review of our data and the literature demonstrates that missense mutations are mainly localised in the transmembrane domain or neighbouring regions, suggesting that they interfere with the channel function. However, further studies are needed to better understand their effect on protein folding and functioning (Fig. 6E).

4.2. Clinical aspects

Patients with homozygous or compound heterozygous ANO5 mutations presented with a homogeneous clinical phenotype that was independent on the kind of mutation (Table 2). All patients were male except one, and the only affected woman in the study was still completely asymptomatic at the age of 47 years. Two asymptomatic women have been described in the literature who, at the ages of 61 and 68 years, presented only with increased CK as a symptom. All patients were male except one, and the only affected woman in the study was still completely asymptomatic at the age of 47 years. Two asymptomatic women have been described in the literature who, at the ages of 61 and 68 years, presented only with increased CK as a disease sign [1]. These data suggest a lower incidence in this gender and the potential presence of protective factors in females.

Disease onset generally occurred in adulthood at a mean age of 37.2 ± 7.1 years (range 28–47 years). Symptoms at onset were mainly myalgia, cramps, and mild weakness; episodes of myoglobinuria occurred in three of the six patients. Overall CK values were mildly or significantly increased (values between 2-fold and 32-fold of normal) and disease progression was slow. All patients were ambulant at the last evaluation (age 47.2 ± 3 years). The majority of patients participated in physical activity at a competitive level during childhood or in physically demanding work.

Concerning muscle involvement, patients showed a peculiar muscle hypotrophy affecting primarily the quadriceps and posterior compartment of the thigh and calf. Three patients (II.1, II.2, and III.1) exhibited calf pseudo-hypertrophy (Fig. 4). Muscle weakness usually affected the quadriceps and hamstrings, preserving the distal muscles. Only two patients presented with mild weakness of the upper limbs. Cardiac or respiratory involvement was not described.

4.3. Muscle biopsy features

Muscle biopsy findings in homozygous and compound heterozygous patients were also quite similar. All probands were subjected to at least one muscle biopsy of the brachial biceps, which generally showed mild myopathic changes with nuclear centralisations, minimal fibre splitting, and necroses. Only one patient’s biopsy showed (II.2) a mild increase in connective tissue (Fig. 3A). Two patients (I and III.1) underwent a second biopsy of the quadriceps femoris. In both specimens the histological analysis demonstrated a more severe pattern, with striking connective tissue substitution, compared to the biopsy performed on the biceps brachii. These data suggest that the site of muscle biopsy may influence the results, with the upper limb muscles being less clinically affected and, therefore, showing a milder pattern. This difference possibly could lead to the exclusion of a diagnosis of muscular dystrophy in some cases.

Protein expression analysis can also be misleading in some cases. Two patients (patient III.1 and IV) had a partial reduction of signal in assays with antibodies against dystrophin. Also, the calpain-3 reduction observed in four patients seemed to be a secondary deficiency, as the calpain-3 gene analysis was negative.

To date, limited ultrastructural data are available. Existing data show multifocal loss of the sarcolemmal membrane [1,9] without vesicle accumulation, and reduplication and thickening of the basal lamina membrane [7]. The electron microscopy study performed in two patients in this study confirmed these data, demonstrating mild myopathic changes, such as duplication of the basal lamina, which had thickened with flocculent aspect, and focal loss of the plasma membrane (Fig. 3C–F).

4.4. Muscle imaging

CT and MRI studies of the muscle were performed in these patients as clinical follow-up studies. The retrospective analyses of these data provided some insight into the preferential distribution of tissue damage in LGMD2L. In our sample, the posterior compartment of the thigh seemed to be more involved; in later stages also gastrocnemius and soleus muscles showed a severe degree of muscle substitution. Among the muscles of the anterior compartment, only the quadriceps muscles showed changes, with a patchy pattern (Fig. 5). These data correlate with the results of clinical evaluations and confirm what has been previously reported [8,11]. Interestingly, the data available for patient I trace the evolution of muscle damage over many years, showing progressive fibro-adipose substitution, predominantly in the posterior compartment.

5. Conclusion

LGMD2L is a rare autosomal recessive form of LGMD in the Italian population, accounting for only 2% of...
diagnosed LGMDs. This form is characterised by high genetic heterogeneity. However, some common clinical, morphological, and imaging findings could be useful in differential diagnosis. Further studies involving both ultrastructural and functional analyses are necessary to better understand the pathogenesis of this form of muscular dystrophy.

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