The Italian LGMD registry: relative frequency, clinical features, and differential diagnosis

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Abstract

Introduction Limb girdle muscular dystrophies (LGMDs) are characterized by high molecular heterogeneity, clinical overlap, and a paucity of specific biomarkers. However, their molecular definition is fundamental for prognostic and therapeutic purposes. *Methods* We created an Italian LGMD registry that included 370 molecularly defined patients. We reviewed detailed retrospective and prospective data and compared each LGMD subtype for differential diagnosis purposes. *Results* LGMD2A and 2B are the most frequent forms in Italy. The ages at disease onset, clinical progression, and cardiac and respiratory involvement can vary greatly between each LGMD subtype. In a set of extensively studied patients, targeted next-generation sequencing (NGS) identified mutations in 36.5% of cases. *Conclusion*: Detailed clinical characterization combined with muscle tissue analysis is fundamental to guide differential diagnosis and to address molecular tests. NGS is useful for diagnosing forms without specific biomarkers, although, at least in our study cohort, several LGMD disease mechanisms remain to be identified.

Key words: Limb girdle muscular dystrophy, differential diagnosis, natural history, next-generation sequencing, genotype-phenotype correlations

INTRODUCTION

Limb girdle muscular dystrophies (LGMDs) are heterogeneous genetic disorders characterized by progressive muscle impairment, predominantly involving proximal and limb girdle muscles with onset after independent ambulation is achieved, and by histological signs of degeneration and regeneration in muscles [1-2]. The clinical spectrum of LGMDs can vary from more severe infantile forms with early loss of independent ambulation to milder adult-onset and slowly progressive weakness. Cardiac and respiratory involvement can be variably present, and cognitive impairment is usually absent. The estimated incidence of LGMDs in Northern England is 2.27/100000 [3], but updated epidemiological data in Italy are lacking. The clinical heterogeneity of LGMDs is generally associated with high molecular variability, and the molecular classification of LGMDs over the last 2 decades has been revised continuously [4-7].

Historically, the rate of discovery of the first genes related to LGMDs was particularly slow and was predominantly based on homemade algorithms, candidate gene sequencing, and linkage analysis. Recently, advanced genetic diagnostic procedures and high-throughput technologies such as whole-exome sequencing (WES) and next-generation sequencing (NGS) associated with bioinformatics tools and gene expression pattern analysis [8-9] have facilitated identification of new causative genes and sped up the diagnostic process. These techniques enabled the diagnosis of at least 5 new LGMD forms in the last 3 years: *DNAJB6, HNRPDL, TNPO3, ISPD,* and *GMPPB* [10-15].

Canonical categorization of LGMDs divides them into autosomal dominant (AD) forms, indicated as LGMD type 1, and autosomal recessive (AR) forms, indicated as LGMD type 2. Among these 2 groups, each LGMD type is indicated with regard to the affected protein and the corresponding gene with a letter given in the order of gene mapping [16]. To date, many LGMD forms have been described, and 29 different molecularly proven forms, including 22 AR and 7 AD, have been classified.

Differential diagnosis between the different forms is difficult, because the initial clinical presentations and the muscle biopsy patterns can be similar. Moreover, so far, no specific immunohistochemical/biochemical tests have been defined to identify the specific protein defects of many of the recently described forms. Some clinical data such as creatine kinase (CK) levels, the clinical and muscle magnetic resonance imaging pattern of muscle involvement, and the presence and age of onset of cardiac and respiratory impairment are fundamental clues to guide the differential diagnosis. Therefore, a detailed knowledge of these aspects, which can only be obtained through population studies, is needed to improve the rate of detection of mutations.

The collaboration between 12 Italian centers specialized in the diagnosis and treatment of neuromuscular diseases enabled collection of clinical, morphological, and molecular data of a large cohort of patients affected by LGMDs. Our aim was to define the spectrum of genetic heterogeneity of LGMDs and the relative frequency of the different forms in the Italian population. Furthermore, the aim of the study was to provide a broad clinical characterization of these forms to define specific clinical features that can guide the differential diagnosis and can delineate the natural history of each subtype. Interestingly, a subgroup of undiagnosed LGMD patients was also analyzed by targeted NGS to detect the causative gene. Finally, the molecular characterization of a large cohort of LGMD patients will aid in the design of natural history studies and therapeutic trials.

MATERIALS AND METHODS

Patient selection

Patients were selected according to the presence of at least 2 of the following inclusion criteria: 1) clinical phenotype compatible with a diagnosis of LGMD according to previously published diagnostic criteria [2]; 2) myopathic or dystrophic muscle biopsy findings; and 3) presence of a disease-causing mutation in 1 of the LGMD genes.

Exclusion criteria were as follows: a different clinical presentation from that of LGMDs (e.g., distal phenotype onset), as well as genetic and/or histopathological findings suggestive of other neuromuscular diseases. Original written informed consents were obtained and stored, according to the declaration of Helsinki, from all subjects or their guardians at the time of the first evaluation, with explicit consent for future use of their data for research purposes. The protocol was also approved by the Ethics Committees of each participating institution.

The data were collected in 12 tertiary care Italian neuromuscular centers.

We identified a series of 599 patients belonging to 483 distinct families. Among these patients, a defined molecular diagnosis was achieved in 370 subjects (187 males and 183 females) belonging to 285 different families (59% of probands). Most (91%) subjects were Italian, but 31 subjects live in Italy but are of foreign origin, originating from Albania, Argentina, Australia, Bangladesh, China, Eritrea, Greece, Macedonia, Morocco, the Middle East, Romania, Slovenia, Sri Lanka, Switzerland, Tunisia, or Venezuela.

Clinical data collection

Detailed clinical information was collected for each patient. We collected data regarding family history, consanguinity between parents, age at disease onset, and age at diagnosis. We considered the appearance of symptoms due to easy fatigability, muscle weakness, inability to run, difficulty walking, and development of cardiac or respiratory involvement at disease onset. The age at diagnosis was defined as the age at which the patient came in for medical attention that led to an LGMD diagnosis.

In all patients, we recorded CK levels at disease onset and during disease progression at least every year, whenever possible. In the majority of patients, an electromyographic (EMG) examination performed at the time of the first evaluation was available.

The clinical symptoms and progression of the disease were studied through periodic neurological visits that included complete neurological examinations and functional tests. Specific features,

such as selective muscle atrophy, hypertrophy or pseudo-hypertrophy, scoliosis, winged scapulae, and tendon contractures, were recorded. The mean follow-up period was 17 ± 11.6 years.

The strength of each muscle group was assessed in all patients according to the Medical Research Council (MRC) Scale, with scores ranging from 0 (no contraction) to 5 (normal strength). Disease progression was evaluated by the loss of independent ambulation and using the modified scale of Gardner-Medwin and Walton [17]. In several patients, motor function was assessed using the motor function measure scale (MFM) and the 6-minute walk test (6MWT).

Cardiac and respiratory functions were assessed at disease onset and annually during follow-up. The cardiac evaluation included electrocardiogram (ECG), Holter-ECG, and echocardiogram. The following data were collected: ejection fraction (EF), shortening fraction (SF), and any sign of cardiac impairment such as hypokinesia, ventricular dilatation, left ventricular hypertrophy, and/or significant arrhythmias. Also, the presence of non-significant arrhythmias, including right bundle branch block (RBBB) and supraventricular or ventricular ectopic beats, was recorded. The onset of cardiac involvement was defined as the onset of cardiac symptoms, reduction of EF < 50%, appearance of hypokinesia, ventricular dilatation, or left ventricular hypertrophy on echocardiography or the beginning of cardiac therapy.

Respiratory function was investigated by spirometry and overnight oximetry. In particular, forced vital capacity (FVC), forced expiratory volume (FEV1), and the age at which the patient started to use non-invasive or invasive ventilation were recorded. The onset of respiratory involvement was defined as deterioration of spirometry (FVC and FEV1 <70%), appearance of nocturnal desaturation on overnight oximetry or start of non-invasive or invasive ventilation.

Muscle biopsy: morphological, immunohistochemical, and Western blot analysis

Most of the patients underwent a diagnostic muscle biopsy, usually at the initial phase of the disease, after written informed consent was obtained. The biopsy was performed mainly in the

biceps brachii or the quadriceps. All of the laboratories involved in biopsy analyses belonged to the Italian Association of Myology and shared several standard operating procedures. The morphological and immunohistochemistry (IHC) analyses were performed in accordance with standard procedures [18]. In detail, 4 digitized non-overlapping consecutive pictures were taken in each biopsy from 4 random areas on the same cryostat section using an optical microscope (model-Leica DC200 equipped with a camera and the image analyzer software IM50) at 20x magnification. The examination of each picture allowed for evaluation of the parameters for each biopsy. The presence of fibro-fatty replacement was evaluated using a scale with a short range of evaluation from + to +++. We considered inflammatory cells as only the cellular infiltrates present at the interstitial level not related to muscle necrotic fibers. Necrotic fibers were described using morphological evaluation criteria in agreement with Dubowitz V [18]. The presence of dystrophinopathy was excluded using 3 monoclonal antibodies against Mid-Rod, NH2, and COOH dystrophin domains both for IHC and Western blot (WB) analyses (Novocastra, Newcastle upon Tyne, UK) [19]. The main proteins involved in muscular dystrophies were analyzed using monoclonal antibodies against α-sarcoglycan, β-sarcoglycan, γ-sarcoglycan, δ-sarcoglycan (Novocastra), dysferlin, α-dystroglycan (anti-α-Dystroglycan Antibody, clone VIA4-1 Millipore 05-298), caveolin-3 (Transduction Laboratories, Lexington, KY), telethonin (Santa Cruz Biotechnology, Santa Cruz, CA), emerin, and merosin [mouse anti-human Merosin (M-chain) Chemicon MAB1922, reacting with the 80 kDa fragment of the M-chain of human merosin], as described in the literature [20]. Alterations in the expression of sarcoglycans, α-dystroglycan, caveolin-3, and merosin detected by IHC were confirmed by WB analysis (WB) using the same monoclonal antibodies. Furthermore, WB analysis was used to detect the reduction of calpain-3 and dysferlin [21]. IHC and WB results were classified using 3 grades: normal, partial deficiency, and absence. The levels of protein reduction were related with clinical severity (age at onset) and genetic results. In a small proportion of cases, we performed a

semiquantitative evaluation by comparing the patient samples with control groups and normalizing the values to the amount of myosin present in the tissue.

Molecular analysis

Direct sequencing of candidate genes

Genetic analysis was performed in all patients and was directed according to the clinical and pathological characteristics as far as the presence of a specific protein defect. Using a candidate gene approach, we studied the following genes involved in autosomal dominant and recessive LGMDs: *MYOT, LMNA, CAV3, DNAJB6, TNPO3, CAPN3, DYSF, SGCA, SGCB, SGCG, SGCD, FKRP, ANO5, FKTN, LARGE, POMT1, POMT2, POMGnT1, ISPD, LAMA2,* and *GMPPB.* Total DNA was extracted from peripheral blood samples, which were obtained after written informed consent in accordance with standard procedures approved by the local ethics committee, and collected in tubes containing EDTA. The samples were then stored at -20°C until the time of processing. For each gene, all exons and adjacent intronic regions were amplified by polymerase chain reaction (PCR) using genomic primers and conditions previously described in the literature. The PCR products were studied by direct sequencing (ABI PRISM 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA); electropherograms were analyzed using the program SeqScape Analysis 2.5 (Applied Biosystems) and compared with reference sequences present in the database. This method allows for detection of small 1-3 nucleotide dup/dels. Furthermore, a cGH array was performed in patients with single heterozygous mutations in genes causative of autosomal recessively transmitted LGMD. The mutations identified were named according to the Leiden Muscular Dystrophy database (http://www.dmd.nl). The nature of the new disease-causing mutations was confirmed by screening healthy control subjects. The parental origin of each mutation was demonstrated by analysis of the genomic DNA of the parents, when available. The effect of putative splicing mutations was investigated by studying the transcript.

The mutations identified were divided into in-frame mutations and truncating mutations. In-frame mutations include deletions/duplications that maintain the reading frame and missense mutations that cause amino acid substitutions. Truncating mutations include nonsense mutations, out-of-frame deletions/insertions/duplications and splicing alterations that prevent the expression of the protein or that lead to the synthesis of a truncated protein. Mutations in patients who carried in-frame and truncating mutations in compound heterozygosity were defined as compound mutations.

Next-generation Sequencing analysis

A subgroup of patients was studied using an NGS targeted platform (HaloPlex Target Enrichment, System Agilent Technologies, Santa Clara, CA, US). This platform (Motorplex) allows for the simultaneous capture of the coding regions of 98 genes that are universally considered to be genetic causes of non-syndromic myopathies [22]. For each locus, all predicted exons and at least 10 flanking nucleotides were always included in the electronic design by the custom NGS Agilent SureDesign webtool. Sequencing and bioinformatics analyses were performed at the Telethon Institute of Genetic and Medicine (TIGEM) in Pozzuoli using an Illumina HiSeq1000 device. Candidate variants called by Motorplex were confirmed by direct sequencing and by analyzing segregation within other family members. Furthermore, they were compared with the clinical and pathological data, and protein IHC analysis was performed in most subjects to confirm the diagnosis.

For this analysis, we selected a well-defined group of 58 LGMD subjects (52 probands). In all of these patients, the candidate genes had been previously screened according to the clinical characteristics and the specific protein defects at muscle biopsy.

Statistical Analysis

The non-parametric Mann-Whitney test was used to assess the correlations between genotype and phenotype, particularly between the type of mutation/age at onset, extent of protein defect/age at onset, and gender/age at onset. Furthermore, a regression analysis was performed to detect whether some of the proteins or clinical characteristics predicted a specific LGMD form. For all statistical tests, the threshold of significance was set at P <0.05. The odds of maintaining independent ambulation in the course of the disease was assessed by Kaplan-Meier analysis.

RESULTS

We collected data from 599 patients (483 probands) from 12 different neuromuscular centers. The sample is representative of the LGMD population in Italy, because the referral centers cover the entire nation. The majority of the patients were of Italian nationality (91%).

Patient data are summarized in Table 1. The description of clinical and instrumental features will be focused on the group of patients with a definitive molecular diagnosis (370 patients; 285 probands), representing 59% of the entire cohort.

Relative frequency of the different LGMD forms

The relative frequency of the LGMD subtypes is shown in Figure 1. Autosomal recessive forms (LGMD2) were the most frequent forms (84% of patients and 89% of probands), whereas autosomal dominant forms (LGMD1) affected 16% of patients (11% of probands). The most frequent forms were LGMD2A (24.7% of patients and 30% of probands) and LGMD2B (23.8% of patients and 22.6% of probands), followed by the sarcoglycanopathies, which together represent 20.1% of the genetically diagnosed patients (21.3% of probands). Within the sarcoglycanopathies, LGMD2D was the predominant form (46.7%). Thirty-six patients (9.7%) were affected by LGMD2I, whereas 14 were diagnosed with LGMD2L (3.8%). Mutations in other LGMD genes were detected in only a few patients.

Among the autosomal dominant forms, the most common was LGMD1C (63.5% of patients with LGMD1).

Patients carrying mutations in the *MYOT, TNPO3,* and *GMPPB* genes were absent in this cohort.

Gender effect

In the majority of LGMD subgroups, both genders were affected equally (Table 1). A higher prevalence of male patients has been observed in LGMD2L (M:F 11:3; 3.7), LGMD1B (M:F 11:8; 1.4), and LGMD1C (M:F 24:16; 1.5).

Furthermore, males frequently have an earlier onset of symptoms. In LGMD1B, the onset in women occurs later than in men (47.0 ± 16.2 years vs. 23.7 ± 13.9 years, $P = 0.031$). This effect also occurs in patients with LGMD1C (28.1 ± 15.9 years vs 22.7 ± 19.1 years, $P = 0.187$), and in subjects suffering from LGMD2C (14.4 ± 4.6 years vs 5.3 ± 2.5 years, $P = 0.003$) and LGMD2D (22.1 ± 16.3 years vs 9.3 ± 5.2 years, $P = 0.04$).

Muscle biopsy findings

Muscle biopsies were performed in all patients, with the exception of family members of previously diagnosed patients. We collected data from 242 biopsies and analyzed the following previously described parameters: increase in connective tissue, necrosis, and signs of inflammation. The majority of the biopsies showed an increase in connective tissue (71%; 173/242 samples) [Supplementary Figure 1A, available online].

Sixty-nine biopsies did not show an increase in connective tissue. This finding occurred mainly in biopsies from patients suffering from LGMD1B (70% of LGMD1B biopsies), LGMD1C (40%), LGMD2D (36%), and, to a lesser extent, in LGMD2I (30%), LGMD2B (27%), LGMD2A (22%), LGMD2E (20%), and LGMD2L (15%) [Supplementary Figure 1A]. The biopsies with normal connective tissue had often been performed at the beginning of the disease (within 5 years from

the onset of muscle symptoms) or even in asymptomatic or mildly symptomatic patients (93% of biopsies with normal connective tissue in LGMD2B patients, 88% in LGMD1C, 83% in LGMD2I, and all of the biopsies in LGMD2E). Interestingly, in 17 patients (8 LGMD2A, 3 LGMD1B, 1 LGMD2B, 2 LGMD2D, 1 LGMD2I, 1 LGMD due to *LAMA2* mutations, and 1 LGMD due to *POMT2* mutations), no increase in connective tissue was observed even if biopsies were collected many years after disease onset (up to 33 years). Furthermore, in LGMD2L, the morphological picture varied depending on the muscle biopsied, because lower limb muscles appeared to be more affected than upper limb muscles.

Also, the degree of connective tissue increase varied among the different forms: LGMD2A, LGMD2C, and LGMD2E had more severe connective tissue infiltration; LGMD2B, LGMD2D, LGMD2I, and LGMD2L had an intermediate pattern; and LGMD1B and LGMD1C showed only mild connective tissue replacement.

Some LGMDs had a high number of necrotic fibers. This aspect was most evident in the forms with severe connective tissue replacement, such as LGMD2B (75%), LGMD2A (65%), and sarcoglycanopathies (62%), but was also present in LGMD2I (60%) and LGMD2L (58%). By contrast, LGMD1B (11%) and LGMD1C (32%) were the forms with less prominent myofiber necrosis [Supplementary Figure 1B].

Inflammatory cells and interstitial infiltrates were particularly frequent in subjects affected by LGMD2A (25%), LGMD2I (33%), LGMD2B (38%), and LGMD2E (50%), and they were completely absent in patients with LGMD1B, LGMD1C, and LGMD2L (except for 1 patient) [Supplementary Figure 1C].

Protein analysis by IHC and WB generally allowed detection of the reduction of specific protein(s). Interestingly, primary calpainopathy generally presents with a reduction of the full length 94 kDa band and a variable decrease of the 30 kDa band observed with the Novocastra monoclonal antibody. Secondary calpain-3 deficiency was found in 41% (16/39) of biopsies from LGMD2B patients and was also found less frequently in biopsies of patients with LGMD2I (n=2),

LGMD2L (n=3), LGMD2D (n=1), LGMD2E (n=1), and LGMD due to merosin deficiency (n=1). Mild dystrophin deficiency was detected by IHC and/or WB analysis in 32 patients (1 LGMD2A, 1 LGMD2B, 5 LGMD2C, 12 LGMD2D, 6 LGMD2E, 2 LGMD2F, 2 LGMD2I, and 3 LGMD2L). In the remaining patients, proteins not directly involved in the pathogenesis showed infrequent and mild reduction.

Molecular aspects

We identified 260 different mutations in the investigated genes. In particular, there were 158 in-frame mutations (148 missense substitutions and 10 in-frame deletions) and 102 truncating mutations (21 nonsense mutations, 54 small deletions and duplications that alter the reading frame, and 27 splicing site mutations) [Figure 2].

In LGMD1C (92%), LGMD2A (68%), LGMD2D (76%), and LGMD2I (75%), there were more in-frame mutations, whereas, in the other forms, there were an equal number of in-frame and truncating mutations. In general, mutation transmission confirmed the expected inheritance mode for each LGMD. However, there was at least 1 exception in a patient suffering from LGMD caused by lack of caveolin-3 who carried a missense homozygous mutation (c.277G>A, p.Ala93Thr, exon 2). This mutation had been previously described. The parents of the patient, who are heterozygous carriers of the mutation, were asymptomatic. This case emphasizes the possibility of sporadic cases with caveolin-3 deficiency and autosomal recessive inheritance.

Most mutations were unique with few exceptions. A total of 56% of the probands affected by LGMD2I carried the mutation c.826C>A (p.Leu276Ile) in exon 4 of the *FKRP* gene [23]. However, for other LGMD forms, we also observed a preponderance of the following mutations: c.136G>A (32% of alleles) in *CAV3*, c.525delT (40% of alleles) in *SGCG*, c.850C>T (30% of alleles) in *SGCA,* and c.377_384dup (28% of alleles) in *SGCB*. The mutation c.550delA (p.Thr184fsX36) in exon 4 of *CAPN3* was found in 7% of the alleles of LGMD2A probands.

Approximately 70% of *CAPN3* mutations are located in 8 of the 24 exons of the gene (exons 11, 4, 10, 13, 1, 21, 5, and 6) [24]. Furthermore, 91% of SGCG mutations affect 3 exons (exons 6, 3, and 8), 88% of *SGCA* mutations involve exons 7 and 6, and 70% of *SGCB* mutations are located in exon 3.

Next-generation sequencing results

The DNA of a group of 52 LGMD probands (58 patients) without molecular diagnosis was analyzed using an NGS targeted platform (Motorplex). All of these patients had a clinical diagnosis of LGMD. In all cases, their muscle biopsies showed a dystrophic pattern with an increase in connective tissue. The analysis revealed causative gene mutations in 19 probands (22 patients) as follows: *CAPN3* (n=5), *DYSF* (n=3), *ANO5* (n=2), *FKRP* (n=1), *SGCA* (n=1), *POMT2* (n=1), *TTN* (n=1), *LAMA2* (n=1), and *TCAP* (n=1). Furthermore, in 3 probands, we detected mutations in *DMD* (n=1), *COL6A3* (n=1), and *GAA* (n=1), and these patients were excluded from the LGMD cohort. The 5 LGMD2A patients had been previously studied by direct sequencing of the *CAPN3* gene, but the analysis revealed only 1 heterozygous mutation. All patients had a dystrophic muscle biopsy with an absence or severe reduction of calpain-3 observed by WB analysis, and NGS allowed detection of the second mutation in the *CAPN3* gene. NGS also revealed the second mutation in the 3 LGMD2B subjects. In the other patients (patients harboring *ANO5, TTN,* or *TCAP* mutations), no specific protein test was available at our laboratory or no muscle tissue was available for further tests (*FKRP, SGCA,* and *POMT2)*. However, previous studies in these patients showed a dystrophic pattern. Overall, the NGS approach was shown to be useful and resulted in a diagnosis in 36.5% of the patients analyzed.

Clinical aspects

Age of onset

The age of onset was highly variable and ranged from the first to the fifth decades of life (range 1-65 years) [Figure 3 A]. The forms with earlier onset were LGMD2E (mean onset age 6.4 ± 5.2 years), LGMD2C (10.1 ± 5.9 years), and some dystroglycanopathies (onset at ages 2, 4, and 23 years for LGMD due to *POMT2* mutations and onset at age 5 years for LGMD due to *ISPD* mutations). The forms with later onset were LGMD1B (mean onset age 35.3 ± 18.8 years) and LGMD2L (36.0 ± 13.2 years). Between these 2 extremes, the other forms sorted from the earliest onset to the latest onset are as follows: LGMD2D (mean onset age 15.5 ± 13.4 years), LGMD2A (16.7 ± 11.1 years), LGMD2I (18.1 ± 15.7 years), LGMD1C (24.4 ± 18.0 years), and LGMD2B (25.1 ± 11.3 years) [Figure 3 B]. Some cases of LGMD2A (n=3), LGMD2B (n=1), LGMD2D (n=1), and LGMD2I (n=1) had onset during early infancy (ages 1-2 years).

Clinical presentation and muscle involvement.

Patients came to medical attention for the following reasons: onset of mild proximal weakness (199 patients), hyperCKemia (79 patients), cardiac involvement (4 patients), or cramps and myalgias (10 patients). Weakness, when present, most frequently involved the lower limbs and extended during disease progression to the upper limbs. It is important to note that 31 patients did not have muscle weakness. These patients were family members of other affected individuals (n=12) or presented for clinical attention with isolated hyperCKemia (3) or clinical features such as rippling and calf hypertrophy (3). Thirteen patients were presymptomatic children assessed at an earlier age than the mean age of onset of the subgroup of LGMD defined by their mutational status. Distal weakness was more frequently detected, also earlier than expected, in some forms, in particular LGMD2B (n=18), LGMD2L (n=3), LGMD1B (n=2), LGMD2A (n=3), LGMD2D (n=1), and LGMD2C (n=1); however, in advanced stages, all patients developed involvement of the distal extremities to varying degrees.

Muscle atrophy/hypertrophy occurred in a different pattern in each LGMD. Calf pseudohypertrophy was frequently found in patients suffering from sarcoglycanopathies

(LGMD2C 5/10, LGMD2D 17/28, and LGMD2E 15/18) and in patients with LGMD1C (21/28), LGMD2I (14/30), and LGMD2L (4/12), sometimes with asymmetric involvement. Hypertrophy of the quadriceps femoris muscle was observed in a few patients with LGMD1C (n=7), and macroglossia was observed in some patients affected with LGMD2I (n=2) and LGMD2E (n=3). Subjects suffering from dysferlinopathy frequently had distal lower limb atrophy (17 patients in our sample).

Tendon contractures were common and appeared at early stages in patients with LGMD2E (15/20, 75%), LGMD2A (42/66; 64%), and LGMD2C (6/10, 60%) but were even found in other forms: LGMD2B (23/53; 43%), LGMD2D (12/29, 41%), LGMD2I (5/18, 28%), LGMD1C (6/23; 26%), LGMD1B (2/10, 20%), LGMD2N (1/3, 33%), and LGMD2L (1/11; 9%). Contractures mainly involved the ankles, elbow flexors, and knees. Scoliosis was more frequent in wheelchair-dependent patients with LGMD2E (9/17, 53%) and LGMD2C (4/10; 40%) and in ambulant patients with LGMD2B (17/47; 36%) and LGMD2A (16/58, 28%). Winged scapulae were observed in 14 patients with LGMD2A and occurred sporadically in the other forms.

Patients affected by LGMD1C typically exhibited cramps, myalgias, and rippling, especially localized to the quadriceps muscles. Three patients with LGMD2L and 3 patients with sarcoglycanopathies had episodes of myoglobinuria.

The EMG examination showed a myopathic pattern in the majority of patients (148/191), which was sometimes associated with neurogenic signs (21/191). In some LGMD patients (11/191), an isolated neurogenic pattern was occasionally observed (LGMD1B, LGMD1C, LGMD2A, LGMD2B, LGMD2D, and LGMD2E). The patients did not show signs of neuropathy, and this pattern was considered part of the LGMD phenotype. Interestingly, in 11 patients, EMG examination was normal. These data are reported in Table 1-2.

Muscle weakness progression.

The majority of LGMD patients had a progressive course, but the evolution was different among each form [Suppl Fig. 2A-C]. LGMD1B, LGMD1C and LGMD2L showed a more benign course: all patients at the time of the last evaluation (mean age 51.1 ± 9.2 years old in LGMD1B; 35.9 ± 19.8 years old in LGMD1C; and 47.7 ± 11.1 years old in LGMD2L) had independent walking function [Suppl Fig 2A]. A more rapid evolution was observed in LGMD2C and LGMD2E, in which 73% and 53% of patients lost independent ambulation during follow-up at 26.0 ± 16.0 years of age (17.2 ± 17.4 years from onset) and 12.4 ± 2.3 years of age (8.5 ± 3.2 years from onset), respectively [Suppl Fig 2B]. Difficulties in postural changes (Walton >5) were found mainly in patients with LGMD2A (65% of patients), LGMD2B (71%) and sarcoglycanopathies (39% of LGMD2D, 61% of LGMD2E and 80% of LGMD2C patients).

Creatine kinase levels.

Serum CK levels at disease onset were elevated in all patients. Higher levels at disease onset were found in sarcoglycanopathies (mean value 7000 U/L) and in dysferlinopathies (mean value 5000 U/L, although some cases had values above 25000 U/L). Conversely, other forms showed very low levels; for example, in LGMD1B patients, serum CK levels never exceeded 1000 U/L [Suppl Fig 3].

Reflecting disease progression, the values greatly decreased over years, and almost all subjects older than 50 years of age had CK values lower than 2000 U/L.

Generally, during disease progression, the higher values were found in LGMD2B (1.5-125x), LGMD2D (1-150x), LGMD2E (1-111x), LGMD2C (2-128x) and LGMD2A (1-108x), whereas the forms with lower values of CK were LGMD2I (1-57x), LGMD2L (2-50x), LGMD1C (1-42x) and LGMD1B (1-4x).

Cardiac involvement

According to our database, the forms that were most frequently associated with heart involvement were LGMD1B (13/13, 100%), LGMD2E (7/20; 35%) and LGMD2I (12/26, 46%). In the remaining forms, cardiomyopathy was observed only in isolated cases [Suppl. Fig. S4A; Table 2A].

Cardiac involvement in LGMD1B was more severe than in other forms and was always present. Seven patients had cardiac arrhythmia, which required the implantation of a cardiac defibrillator, and 3 subjects underwent cardiac transplantation (at the ages of 42, 50, and 58 years). Patients suffering from LGMD2I mainly had ventricular dilatation and/or hypokinesia wall, whereas hypokinetic heart disease was predominant in LGMD2E subjects.

The age at onset of heart involvement varied in the different forms, with a relatively earlier onset in LGMD2E (20.3 ± 4.2 years old) and a later onset in LGMD1B (36.4 ± 10.2 years old) and LGMD2I (31.0 ± 21.7 years old) [Suppl Fig.S4 C; Table 2A].

In patients with LGMD1B, cardiac impairment often preceded skeletal muscle involvement (7/11 patients), whereas cardiac impairment generally followed skeletal muscle involvement in the other forms. In LGMD2E and LGMD2I, cardiac function deteriorated 16.6 ± 9.6 and 2.7 ± 2.1 years after disease onset, respectively.

Respiratory involvement

Respiratory involvement was uncommon in LGMDs and was generally characterized by a restrictive pattern. The deterioration of respiratory function appeared at an average age of 15.6 ± 8.5 years after symptoms onset. This occurred more often in LGMD2I (12/24, 50%) and sarcoglycanopathies (LGMD2C 7/14, 50%; LGMD2D 6/25, 24%; and LGMD2E 8/20, 40%) and less frequently in patients with calpainopathy (11/53, 21%) and dysferlinopathy (7/40; 18%) (Suppl. Fig. 4B, 4D). In some cases, respiratory impairment was very severe and required assisted ventilation, as observed in 3 LGMD2D, 3 LGMD2E, 2 LGMD2B, 1 LGMD2I and 1 LGMD2C

patients. Respiratory dysfunction was very rare in the remaining forms [Suppl. Fig S4 B; Table 2B].

Genotype-phenotype correlations

We identified a correlation between the type of mutation, protein expression and clinical progression. We used the patient's age at disease onset as an indicator of clinical severity, and we observed that truncating mutations were generally associated with more severe protein reduction and clinical presentation. Mutations that preserve the reading frame were usually responsible for milder phenotypes.

In fact, in-frame mutations were generally associated with a later onset than truncating mutations. In particular, this difference was statistically significant in LGMD2A (19.7 ± 11.5 years vs. 12.2 ± 11.2 years, p = 0.02), LGMD2B (30.5 ± 10.3 years vs. 17.7 ± 5.5 years, p <0.0001) and LGMD2E (10.8 ± 5.4 years vs. 4.1 ± 3.7 years, p = 0.007).

LGMD2A, LGMD2B, LGMD2D and LGMD2E patients with protein absence observed by IHC and/or WB assays had a lower age of disease onset than patients with partial deficiency. The difference was statistically significant in LGMD2D (absence of α-sarcoglycan 5.4 ± 2.3 years vs. partial defect 20.0 ± 14.5 years, p = 0.0001) and LGMD2E (absence of β-sarcoglycan 3.1 ± 1.4 years vs. partial defect 13.7 ± 5.7 years, p = 0.015). We also evaluated the predictive value of specific protein and clinical data to unequivocally address the differential diagnosis between the different LGMD forms. According to this analysis, the presence of rippling was a specific indicator of LGMD1C with 100% specificity and 40% sensitivity (p = 0.002). The other clinical and muscle biopsy findings, even if they were particular to certain LGMD forms, did not reach statistical significance.

Discussion

In this study, we present an overview of the clinical and molecular characteristics of a large cohort of LGMD patients who are representative of the Italian LGMD population.

The data collected allowed us to better define the epidemiology, the phenotype and the natural history of each LGMD and to detect the most important parameters that can help the differential diagnosis.

This project was carried out with the collaboration of 12 different Italian centers involved in the diagnosis and cure of neuromuscular diseases. We collected data from 599 patients, and the clinical histories of 370 molecularly confirmed patients were analyzed in detail.

Relative frequency of LGMD in Italy

The collection of a large cohort of patients allowed us to define the relative frequency of genetically diagnosed patients with LGMD in Italy (Fig. 1). According to the data reported in the literature, in our cohort, AR LGMDs were more frequent then AD LGMDs (84% of patients - 89% of probands). Even after the discovery of several new genes, mutations in *CAPN3* and *DYSF*, which cause LGMD2A and LGMD2B, remain responsible for half of the cases in our cohort (30% and 22.6.2% of probands, respectively). Also, sarcoglycanopathies were frequent and affected 21.3% of probands. LGMD2A appears to be the most frequently diagnosed form not only in Italy, as previously reported in the literature [9, 25], but also in the other countries because it has been described as the most common form in populations from Brazil, Japan, the Netherlands, Turkey, Russia and Basque regions [26-31].

By contrast, LGMD2I, common in Denmark and Northern England [16; 32], and LGMD2L, common in Northern Europe [33], affected only 9.8% and 3.8% of patients, respectively, in our sample. In Denmark, LGMD2I is responsible for 42% of patients with AR LGMD (12% of the patients in our sample), followed by LGMD2L (11%), which represents, with LGMD2A, the second most common form of AR LGMD (5% of LGMD2 patients in our sample) [33]. This difference may be partially due to a North-South gradient and to a founder effect for some

mutations in Northern Europe populations. The other dystroglycanopathies were poorly represented in our cohort and affected only 1 *ISPD* subject and 3 *POMT2* patients (now 15, 24, 42 and 48 years old), with childhood onset.

Interestingly, we described 2 families (5 patients) who presented with adult-onset LGMD and carried mutations in the *LAMA2* gene, which is generally associated with Congenital Muscular Dystrophies [34]. Patients affected with AD forms in our cohort carried mutations in the *CAV3*, *DNAJB6* and *LMNA* genes. We did not find mutations in the other LGMD1 genes, which have presently only been reported in a few families worldwide [10-12].

Targeted NGS increased the detection rate of LGMD genetic causative mutations in this cohort, and 36.5% of genetic diagnoses were identified using this technique. This figure does not fully address the potential of targeted NGS in LGMD because the sample analyzed by NGS had been previously extensively studied with other techniques. Therefore, the rate of detection might be higher in a "naïve" LGMD sample. Meanwhile, the rate of detection using 98 genes involved in muscle diseases as a reference does not address the cause of LGMD in 63.5% of patients, confirming the still unexplored genetic causes of this group of disorders.

LGMD differential diagnosis

The clinical findings showed wide variability, both between different forms and within the same LGMD groups. We focused the analysis on the aspects that were specific for each form and that should be evaluated to aid in differential diagnosis.

The study of the natural history and the detailed knowledge of the specific characteristics of each LGMD subtype allowed us to detect the most important clues that could help the clinician in the diagnosis (Figure 4).

From this perspective, the most important parameters were the muscle pathology findings and the clinical presentation, in particular CK levels, age of onset, presence of contractures, and cardiac

and respiratory involvement. Taken together, all of these data can help one choose the molecular tests.

Muscle biopsy can provide important clues for the differential diagnosis. The presence of a dystrophic or myopathic pattern combined with the clinical presentation of limb girdle weakness is highly suggestive of LGMD. However, it should be noted that the absence of an increase in connective tissue does not exclude the diagnosis (Suppl Figure S1A, 29% of biopsies), especially when the biopsy was performed at early stages. LGMD1C and LGMD1B often showed isolated mild myopathic signs. Furthermore, the different degree of connective tissue substitution, the prevalence of necrotic and degenerating fibers and the presence or absence of inflammatory infiltrates can aid in the differential diagnosis (Suppl Figure S1). For example, inflammation was more frequent in dysferlinopathies and calpainopathies (Suppl Fig. S1B).

IHC and WB studies performed on muscle samples allowed us to detect reductions in specific proteins. In some forms, for example sarcoglycanopathies, reduction of the specific protein complex strongly correlated with mutations (100% of our patients with mutations in sarcoglycan genes had corresponding protein deficiency measured by IHC or WB assays). However, sometimes this analysis can be misleading, as demonstrated in by the presence of 3 LGMD2A subjects with normal calpain-3 expression. Furthermore, some proteins can be secondarily reduced. In this cohort, calpain-3 deficiency was found in patients with LGMD2B, sarcoglycanopathies, LGMD2I, LGMD2L, and LGMD due to merosin deficiency, whereas dystrophin deficiency was detected in LGMD2A, sarcoglycanopathies, LGMD2I, and LGMD2L. When a deficiency of glycosylation of α-dystroglycan is detected, the molecular diagnosis is difficult, because in recent years the discovery of genes responsible for these α-dystroglycanopathies has increased exponentially. However, according to our experience and to the epidemiological data, the LGMD2I form is more frequent than the others, and the *FKRP* gene should be investigated first. Unfortunately, for some subtypes, for example LGMD2L, no protein

test is available. In these cases, the clinical characteristics are fundamental to aid in the molecular analysis.

Sarcoglycanopathies, with the exception of LGMD2D, are generally the most severe forms, with early onset and rapid progression, leading to loss of independent ambulation in the first 2 decades of life in a high proportion of patients.

According to the age of onset, we can subdivide the LGMD population into different groups (Figure 3). Forms with onset before age 10 years are mainly sarcoglycanopathies, dystroglycanopathies, and some cases of calpainopathies. Generally, a significant proportion of LGMD2A patients have an age of onset <20 years, whereas the onset of symptoms for patients with dysferlinopathy occurs between ages 11 and 40 years. LGMD1C and LGMD2I may manifest in all age groups, but more frequently occur before age 20 years. Conversely, patients with LGMD1B and LGMD2L develop their first symptoms primarily after age 20 years (Figure 3). Furthermore, in some forms with later onset, few cases of unusually early onset (ages 1-2 years) were observed.

Earlier age of onset often correlated with severe prognosis. The most severe evolution was observed in LGMD2C and LGMD2E patients, who lost ambulation in 73% and 53% of cases and at an average age of 26.0 ± 16.0 and 12.4 ± 2.3 years, respectively (Suppl Figure S2).

Additionally, CK levels reflect the severity of the disease and can contribute to the differential diagnosis. Only CK at onset can be used for this purpose, because, during disease progression, CK values decrease in all LGMD forms. The presence of high CK levels at onset suggests the molecular diagnosis of sarcoglycanopathies and dysferlinopathies, whereas very low levels are frequently found in LGMD1B and LGMD1C [Suppl Figure S3].

Cardiac and respiratory involvement are variably present in LGMD patients and can point toward specific subtypes. Cardiac involvement is constant in LGMD1B and frequent in LGMD2E and LGMD2I [Supplementary Figure S4A]. Given the early onset of heart disease and its severity,

molecular analysis of the *LMNA* gene and a regular cardiologic follow-up should be performed in all family members of patients with LGMD1B. A restrictive respiratory pattern is present in LGMD2I, sarcoglycanopathies and rarely in calpainopathy and dysferlinopathy [Suppl Figure S4B].

The definition of the frequency, characteristics and severity of cardiomyopathy and pulmonary involvement in each form is fundamental to develop guidelines for follow-up and to develop programs for proper cardiac and respiratory monitoring, which allows for establishing timely and adequate medical treatment or surgery [6].

We highlight the contributions of other peculiar clinical characteristics for the differential diagnosis (Tables 1, 2). Sarcoglycanopthies and patients affected by LGMD1C, LGMD2I, and LGMD2L frequently presented with calf pseudohypertrophy. Some subjects carrying mutations in the dysferlin gene developed distal lower limb atrophy, whereas a few patients suffering from LGMD1C and LGMD2E had quadriceps femoris hypertrophy. The early appearance of tendon contractures is mainly found in LGMD2A, LGMD2E, and LGMD2C patients, whereas scoliosis is found in LGMD2E, LGMD2C, LGMD2B, and LGMD2A patients in particular. Episodes of myoglobinuria can be observed in patients with LGMD2L and rippling in *CAV3* forms (P=0.002). Key clinical and paraclinical findings that may aid in addressing a specific diagnosis during clinical work-up are summarized in Tables 1 and 2 and in Figure 4.

Molecular analysis and contribution of NGS

The majority of patients were studied by direct sequencing of candidate genes. Generally, we did not find mutational hotspots, although there were some useful exceptions, as described in the Results section. NGS was found to be a cost-effective method that can be used to study patients for whom the candidate gene approach was unable to reveal mutations. NGS allowed for detection of the mutation on the second allele in AR LGMD and the study of subjects without an

available muscle protein test. Overall, in our sample, NGS allowed detection of mutations in 19 of the 52 probands studied (36.5%).

A detailed knowledge of the clinical characteristics of a large sample of LGMD patients allowed us to define important clues that can be used in the differential diagnosis of LGMD patients and for designing clinical studies of each individual form. Gene product expression and function in skeletal muscle and clinical data should still be combined to choose the molecular tests. New molecular techniques, such as NGS, must be considered in patients for whom the candidate gene approach failed to detect mutations and in forms still lacking a tissue-specific marker.

Abbreviations list

AD autosomal dominant

AR autosomal recessive

CK creatine kinase

ECG electrocardiogram

EF ejection fraction

EMG electromyographic

FVC forced vital capacity

FEV1 forced expiratory volume

IHC immunohistochemistry

LGMD Limb girdle muscular dystrophies

MFM Motor function measure scale

MRC Medical Research Council

6MWT Six-minute walking test

NGS next-generation sequencing

PCR polymerase chain reaction

RBBB right bundle branch blocks

SF shortening fraction

TIGEM Telethon Institute of Genetic and Medicine

WES whole exome sequencing

WB Western blot

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Table 1 Clinical characteristics of different forms of LGMD

	Probands/patients, n	Male/Female	Last evaluation (years)	Onset (years)	CK at onset (UI/L)	CK range (follow-up)
LGMD1B	9/19	11/8	52.0 ± 10.6	35.3 ± 18.8	394 ± 197	1-4x
LGMD1C	21/40	24/16	36.5 ± 20.0	24.4 ± 18.0	1890 ± 1907	1-42x
LGMD1D	1/4	1/3	52.2 ± 11.6	28	1600	4-8x
LGMD2A	86/92	39/53	36.7 ± 15.4	16.7 ± 11.1	4025 ± 3393	1-108x
LGMD2B	65/85	41/44	39.3 ± 11.8	25.1 ± 11.3	5147 ± 5299	1.5-125x
LGMD2C	15/17	8/9	25.1 ± 15.7	10.1 ± 5.9	6388 ± 7640	2-128x
LGMD2D	28/35	18/17	28.9 ± 18.3	15.5 ± 13.4	8011 ± 8651	1-150x
LGMD2E	16/21	10/11	21.5 ± 10.2	6.4 ± 5.2	7922 ± 6797	1-111x
LGMD2F	2/2	2/0	14.0	4.0	20000	100x
LGMD2I	25/36	19/17	38.3 ± 21.1	18.1 ± 15.7	3162 ± 2682	1-57x
LGMD2L	12/14	11/3	47.7 ± 11.1	36.0 ± 13.2	3925 ± 2771	2-50x
LGMD POMT2	3/3	1/2	24.3 ± 11.7	9.7 ± 11.6	5217 ± 1624	17-32x
LGMD LAMA2	2/5	1/4	55± 13.5	17.0 ± 7.5	670 ± 239	2-3x
LGMD ISPD	1/1	1/0	42.0	5.0	ND	3-6x

Table 2A Clinical evolution in different forms of LGMD

	Probands/ patients, n	Ambulation loss (%)	Ambulation loss (age)	Cardiac involvement (%)	Cardiac involvement (age)
LGMD1B	9/19	0/11 (0%)	-	13/13 (100%)	36.4 ± 10.2
LGMD1C	21/40	0/25 (0%)	-	3/23 (13%)	51.0
LGMD1D	1/4	0/4 (0%)	-	0/4 (0%)	-
LGMD2A	86/92	20/65 (31%)	39.9 ± 11.5	4/56 (7%)	40.5 ± 30.4
LGMD2B	65/85	18/60 (30%)	43.1 ± 12.7	2/45 (4%)	41.0 ± 4.2
LGMD2C	15/17	8/11 (73%)	26.0 ± 16.0	3/15 (20%)	20.3 ± 4.2
LGMD2D	28/35	4/28 (14%)	14.3 ± 4.6	3/30 (10%)	25.7 ± 5.5
LGMD2E	16/21	10/19 (53%)	12.4 ± 2.3	7/20 (35%)	20.6 ± 7.9
LGMD2F	2/2	1/1 (100%)	11,0	1/1 (100%)	9.0
LGMD2I	25/36	5/22 (23%)	48.0 ± 19.4	12/26 (46%)	31.0 ± 21.7
LGMD2L	12/14	0/14 (0%)	-	0/11 (0%)	-
LGMD POMT2	3/3	0/3 (0%)	-	0/3 (0%)	-
LGMD LAMA2	2/5	1/5 (0%)	57	0/5 (0%)	-
LGMD ISPD	1/1	0/1 (0%)	-	0/1 (0%)	-

Table 2B Clinical evolution in different forms of LGMD

	Respiratory involvement (%)	Respiratory involvement (age)	Distinctive characteristics
LGMD1B	1/7 (14%)	ND	Arrhythmic cardiomyopathy
LGMD1C	2/17 (12%)	ND	Calf (75%) and quadriceps hypertrophy, rippling, myalgia
LGMD1D	0/4 (0%)	-	-
LGMD2A	11/53 (21%)	37.8 ± 22.8	Winged scapula, tendon contractures (64%)
LGMD2B	7/40 (18%)	34.7 ± 6.7	Distal weakness
LGMD2C	7/14 (50%)	23.2 ± 7.0	Calf hypertrophy (50%), tendon contractures (60%)
LGMD2D	6/25 (24%)	24.8 ± 9.7	Calf hypertrophy (61%)
LGMD2E	8/20 (40%)	20.4 ± 10.7	Calf hypertrophy (83%), macroglossia, tendon contractures (75%)
LGMD2F	1/1 (100%)	14.0	Calf hypertrophy, tendon contractures
LGMD2I	12/24 (50%)	30.4 ± 23.5	Calf hypertrophy (47%)
LGMD2L	0/8 (0%)	-	Myoglobinuria
LGMD POMT2	1/2 (50%)	16.0	Calf hypertrophy, scoliosis
LGMD LAMA2	0/5 (0%)	-	Brain MRI with white matter anomalies, calf hypertrophy
LGMD ISPD	1/1 (100%)	ND	Calf hypertrophy

Figure legends

Figure 1. Relative frequency of LGMDs in the Italian sample. **(A)** Relative frequency of different LGMD forms among probands. (n = 285) **(B)** Relative frequency of different LGMD forms among all patients. (n = 370). LGMDs due to mutations in *CAPN3* and *DYSF* are the most frequent forms.

Figure 2. Mutation distribution in different LGMD forms. The relative proportion of in-frame (in green) and out-of-frame (red and yellow) mutations are detailed for each LGMD form. In the majority of LGMD subtypes. in-frame mutations are more frequent than mutations causing protein loss.

Figure 3. Age of onset in different LGMDs. **(A)** Differential diagnosis of LGMD according to age of onset. The LGMD sample was divided into 4 groups according to the age of onset. For each group. the figure shows the number of patients with each LGMD form. The age of onset can be used to help in the differential diagnosis. **(B)** Mean age at onset for each LGMD form. Each line shows the range of age of onset for each group; in bold the mean age of onset is expressed in years.

Figure 4. Differential diagnosis in LGMDs. The diagram illustrates the main muscle histology and clinical aspects that should be taken into consideration for LGMD differential diagnosis.

A

Probands (%)

0,3% 0,7%
1,0% 0,3% 3,1%
4,2%   7,3%
8,7%   0,3%
0,3% 0,7%
5,6%   29,9%
9,7%
5,2%
22,6%

- LGMD1B
- LGMD1C
- LGMD1D
- LGMD2A
- LGMD2B
- LGMD2C
- LGMD2D
- LGMD2E
- LGMD2F
- LGMD2G
- LGMD2I
- LGMD2L
- LGMD2J
- LGMD2 POMT2
- LGMD2 LAMA2
- LGMD2 ISPD

B

Patients (%)

0,3% 0,8% 1,3% 5,1%
0,3%
3,7%   10,6%
9,6%
0,5%   1,1%
0,3%
5,6%
24,5%
9,3%
4,5%
22,6%

- LGMD1B
- LGMD1C
- LGMD1D
- LGMD2A
- LGMD2B
- LGMD2C
- LGMD2D
- LGMD2E
- LGMD2F
- LGMD2G
- LGMD2I
- LGMD2L
- LGMD2J
- LGMD2 POMT2
- LGMD2 LAMA2
- LGMD2 ISPD
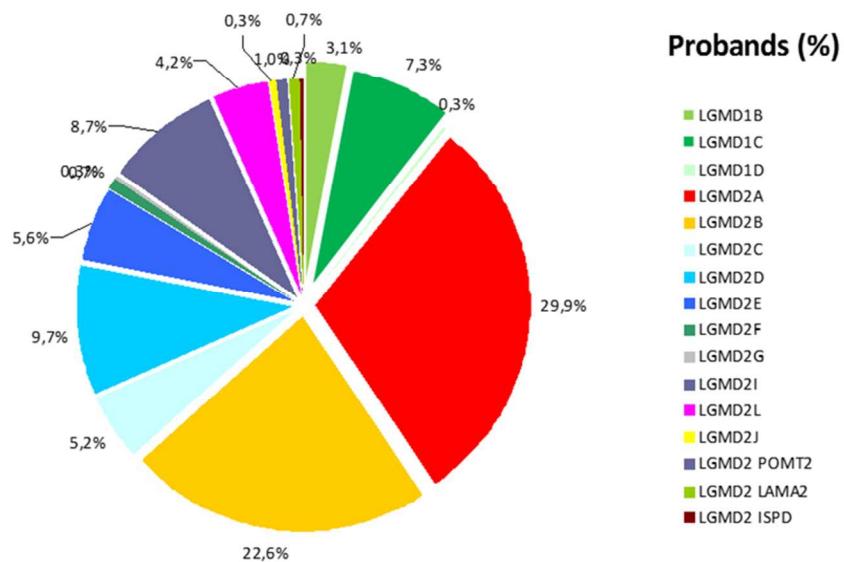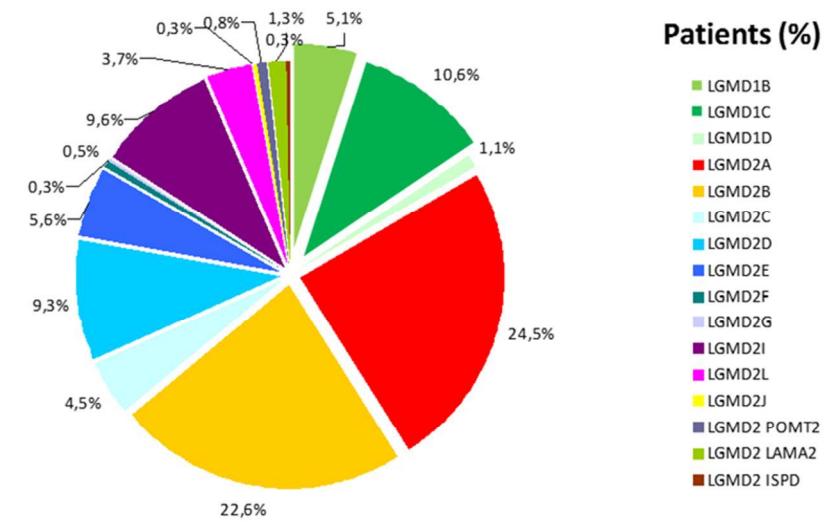
Figure 1. Relative frequency of LGMDs in the Italian sample. (A) Relative frequency of different LGMD forms among probands. (n = 285) (B) Relative frequency of different LGMD forms among all patients. (n = 370). LGMDs due to mutations in CAPN3 and DYSF are the most frequent forms.
80x94mm (300 x 300 DPI)
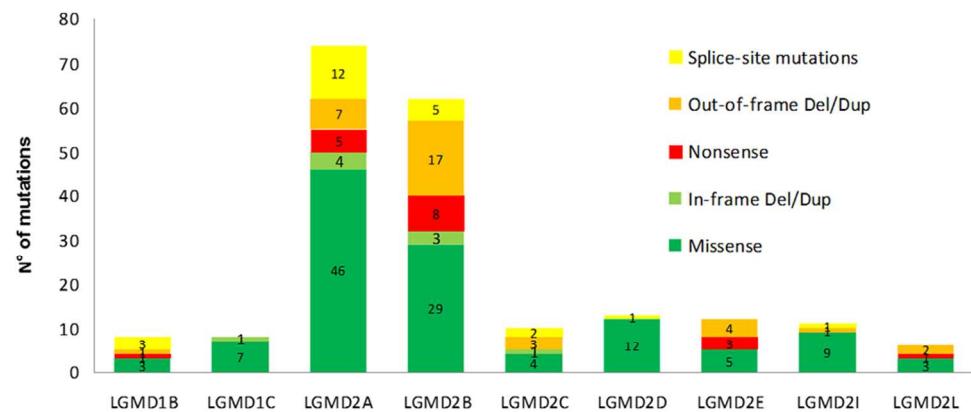
Fig 2. Mutation distribution in different LGMD forms
The relative proportion of in-frame (in green) and out-of-frame (red and yellow) mutations are detailed for each LGMD form. In the majority of LGMD subtypes, in-frame mutations are more frequent than mutations causing protein loss.

90x39mm (300 x 300 DPI)
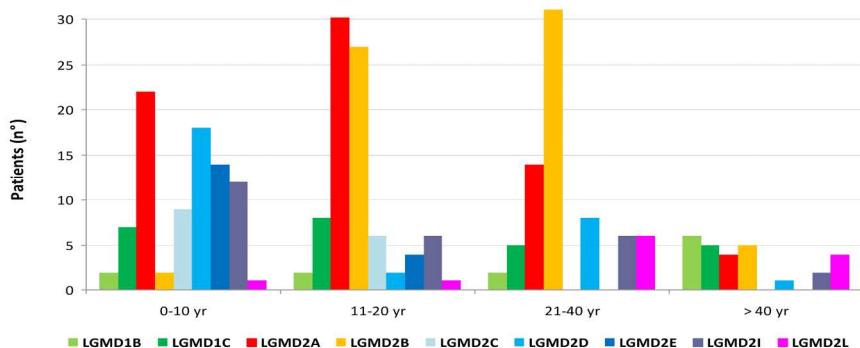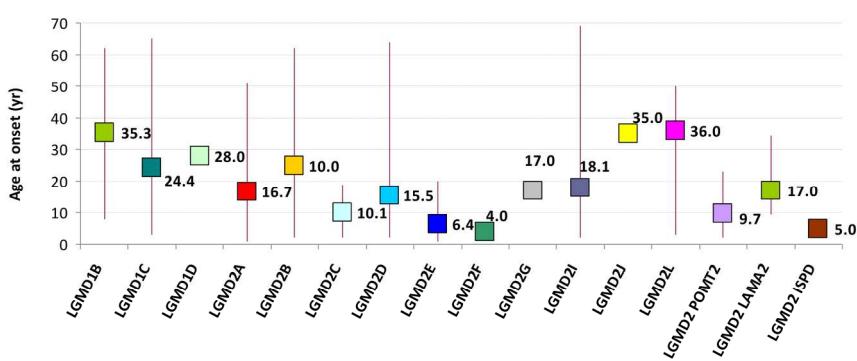
Figure 3. Age of onset in different LGMDs. (A) Differential diagnosis of LGMD according to age of onset. The LGMD sample was divided into 4 groups according to the age of onset. For each group. the figure shows the number of patients with each LGMD form. The age of onset can be used to help in the differential diagnosis. (B) Mean age at onset for each LGMD form. Each line shows the range of age of onset for each group; in bold the mean age of onset is expressed in years.
209x172mm (300 x 300 DPI)
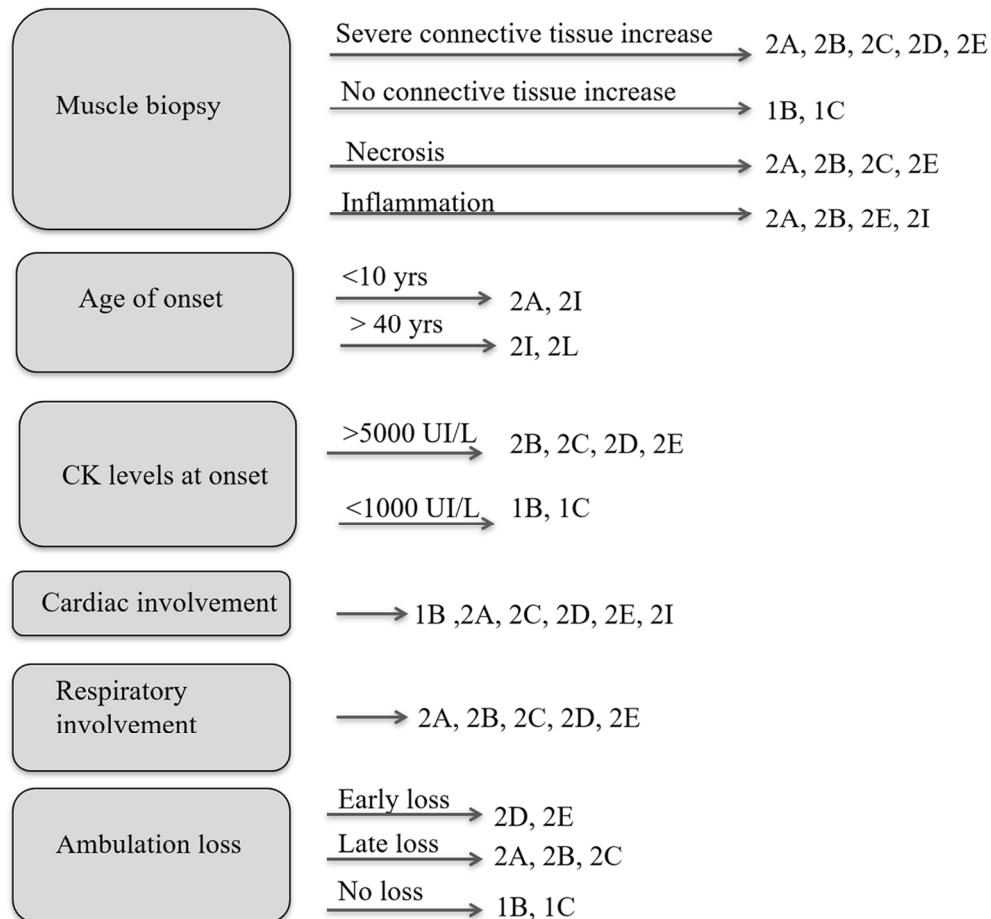
Figure 4. Differential diagnosis in LGMDs. The diagram illustrates the main muscle histology and clinical aspects that should be taken into consideration for LGMD differential diagnosis.
109x103mm (300 x 300 DPI)