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*RESEARCH ARTICLE*

# Clinical, Molecular, and Protein Correlations in a Large Sample of Genetically Diagnosed Italian Limb Girdle Muscular Dystrophy Patients

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**Limb girdle muscular dystrophies (LGMD) are characterized by genetic and clinical heterogeneity: seven autosomal dominant and 12 autosomal recessive loci have so far been identified. Aims of this study were to evaluate the relative proportion of the different types of LGMD in 181 predominantly Italian LGMD patients (representing 155 independent families), to describe the clinical pattern of the different forms, and to identify possible correlations between genotype, phenotype, and protein expression levels, as prognostic factors. Based on protein data, the majority of probands (n = 72) presented calpain-3 deficiency; other defects were as follows: dysferlin (n = 31), sarcoglycans (n = 32), α-dystroglycan (n = 4), and caveolin-3 (n = 2). Genetic analysis identified 111 different mutations, including 47 novel ones. LGMD relative frequency was as follows: LGMD1C (caveolin-3) 1.3%; LGMD2A (calpain-3) 28.4%; LGMD2B (dysferlin) 18.7%; LGMD2C (γ-sarcoglycan) 4.5%; LGMD2D (α-sarcoglycan) 8.4%; LGMD2E (β-sarcoglycan) 4.5%; LGMD2F (δ-sarcoglycan) 0.7%; LGMD2I (Fukutin-related protein) 6.4%; and undetermined 27.1%. Compared to Northern European populations, Italian patients are less likely to be affected with LGMD2I. The order of decreasing clinical severity was: sarcoglycanopathy, calpainopathy, dysferlinopathy, and caveolinopathy. LGMD2I patients showed both infantile noncongenital and mild late-onset presentations. Age at disease onset correlated with variability of genotype and protein levels in LGMD2B. Truncating mutations determined earlier onset than missense substitutions (20 ± 5.1 years vs. 36.7 ± 11.1 years; P = 0.0037). Similarly, dysferlin absence was associated with an earlier onset when compared to partial deficiency (20.2 ± standard deviation [SD] 5.2 years vs. 28.4 ± SD 11.2 years; P = 0.014). Hum Mutat 29(2), 258–266, 2008.** **© 2007 Wiley-Liss, Inc.**

KEY WORDS: limb girdle muscular dystrophy; LGMD; genotype–phenotype

## INTRODUCTION

Limb girdle muscular dystrophies (LGMDs) are a heterogeneous group of inherited progressive muscle disorders affecting predominantly the shoulder and pelvic girdle muscles. There are at least 19 different subtypes of LGMD, seven with an autosomal dominant and 12 with an autosomal recessive pattern of inheritance [Bushby, 1999; Mathews and Moore, 2003; Starling et al., 2004; Guglieri et al., 2005; D'Amico et al., 2006; Jarry et al., 2007; Moore et al., 2006]. The autosomal dominant forms (LGMD1) are relatively rare and represent probably less than 10% of all LGMD cases. Only three LGMD1 genes have been so far identified: they encode for myotilin (MIM# 604103), lamin A/C

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(MIM# 150330), and caveolin-3 (MIM# 601253) [Guglieri et al., 2005]. The genes responsible for autosomal recessive forms (LGMD2) encode highly diverse proteins involved in different aspects of muscle cell biology, such as the sarcolemmal muscle membrane (α-sarcoglycans, *SGCA*, MIM# 600119; β-SG, *SGCB*, MIM# 600900; γ-SG, *SGCG*, MIM# 608896; δ-SG, *SGCD*, MIM# 601411), the membrane repair process (dysferlin, *DYSF*, MIM# 603009), the sarcomere (telethonin, *TCAP*, MIM# 604488; and titin, *TTN*, MIM# 188840), the muscle cytosol (calpain-3, *CAPN3*, MIM# 114240; and *TRIM32*, MIM# 602290), and the glycosylation pathway enzymes (Fukutin related protein, *FKRP*, MIM# 606596; and Protein O-Mannosyltransferase 2, *POMT2*, MIM# 607439) [Guglieri et al., 2005].

These muscular dystrophies are clinically characterized by progressive muscle weakness and atrophy with predominant involvement of scapular and pelvic-girdle, typical facial sparing, and elevated serum creatine kinase (CK) levels. Nevertheless LGMDs usually show a large clinical variability regarding age of onset, patterns of skeletal muscle distribution, heart damage, respiratory involvement, and rate of progression. These diseases result in variable morbidity and disability.

Difficulties in classification are often caused by the relatively common sporadic occurrence of autosomal recessive forms as well as by intrafamilial clinical variability [Mathews and Moore, 2003].

The relative frequency of the different forms of LGMD2 is still under investigation and seems to depend on ethnic clusters and geographic origins; indeed, calpainopathy (LGMD2A) is the most prevalent form in Italy, Brazil, Japan, the Netherlands, Turkey, the Basque region, and Russia [Van der Kooi et al., 1996; Dincer et al.,1997; Urtasun et al.,1998; Passos-Bueno et al., 1999; Pogoda et al., 2000; Chae et al., 2001; Zatz et al., 2003; Fanin et al., 2005], while LGMD2I, caused by mutations in FKRP gene, is the most common form in Denmark and in the North of England [Frosk et al., 2005; Sveen et al., 2006]. Up to now a molecular diagnosis remains elusive in about 30% of LGMD patients.

Herein, we describe detailed clinical, biochemical, and molecular data of a large predominantly Italian population affected with LGMD. Aims of this study were to evaluate the relative proportion of the different types of LGMDs in a large sample of families, to describe their clinical pattern (age of onset, clinical course, rate of progression, skeletal muscle involvement, and heart and respiratory function), and to identify possible correlations between genotype, phenotype, and protein expression levels, as prognostic factors.

## MATERIALS AND METHODS

### Subjects

Patients diagnosed as affected with LGMD were recruited from the neuromuscular clinics of participating institutions. The study included 181 patients (92 males and 89 females) from 155 families; 92% of patients were from Italian families; the remaining included an Italian boy of Gypsy descent and patients from other countries (Switzerland, Algerian, Germany, India, Sri Lanka, and Australia). These latter patients were not considered in the evaluation of the population-based proportion of LGMDs.

The following tissue banks were the main sources of the muscle and DNA samples used in this study: 1) Bank of DNA, Nerve and Muscle Tissues, Department of Neurological Sciences, "Fondazione I.R.C.C.S. Ospedale Maggiore Policlinico, Mangiagalli e Regina Elena," Milan, Italy; 2) Department of Neurosciences, Azienda Ospedaliera Universitaria "G. Martino," Messina, Italy; and 3) Division of Neuromuscular Disorders, Fondazione I.R.C.C.S. Istituto Neurologico Nazionale "C. Besta," Milan, Italy. Written informed consent was obtained (and preserved in original) from all subjects or their caregivers at the moment of primary diagnostic procedures, with explicit consent to future uses for research purpose, according to the Declaration of Helsinki. This protocol was approved by the Institutional Review Boards of participating institutions.

Inclusion in the study was based on fulfillment of the following criteria: 1) a clinical phenotype characterized by progressive muscle weakness and wasting affecting primarily or predominantly shoulder-girdle and pelvic muscles, in keeping with the diagnostic criteria for LGMD [Beckmann et al., 1999]; 2) dystrophic features at the muscle biopsy.

Four patients did not fulfil these criteria because they were asymptomatic at the time of the first clinical evaluation, but were nevertheless included according to elevated serum CK levels, a dystrophic pattern at muscle biopsy, deficiency of one or more proteins implicated in LGMDs at immunohistochemical (IHC) or Western blot (WB) analysis, and mutations in any of the LGMD genes.

Patients were excluded if they had a clinical presentation different from LGMD (for instance, distal dysferlin phenotypes were not included in this cohort), if they presented histopathologic features suggestive of other neuromuscular diseases (e.g. myotonic disorders, facioscapulohumeral dystrophy [FSHD], congenital muscular dystrophy, congenital myopathy, inflammatory myopathy, glycogen or lipid storage myopathy, or mitochondrial myopathy), and if they were genetically diagnosed as affected by any other muscular disorder (such as FSHD, dystrophinopathies, or hereditary inclusion body myopathy).

The patients were interviewed about their family history, presence of consanguinity, clinical history with time and localization of disease onset, and rate of progression. Clinical onset was defined as the time of beginning of symptoms. Earlier symptoms were considered muscle weakness, fatigue, cramps after physical exertion, and tendency to fall. Mean follow-up time for the entire cohort was 13 years (range 1–42 years). At each evaluation, all subjects underwent to a complete neurological examination, to muscle strength evaluation using the Medical Research Council (MRC) scale graded 0 to 5, to a functional assessment by calculating the time of Gowers' maneuver and the time it took the patient to walk 10 meters and climb five steps.

A Gardner-Medwin and Walton modified scale of clinical severity score was used to monitor evolution over time. The features of each category of this scale are as follows: grade 0, preclinical, hyperCKemia, all activities normal; grade 1, normal gait, inability to run freely; grade 2, defect of posture/gait; grade 3, muscle weakness, climbing stairs with support; grade 4, presence of Gowers' sign; grade 5, unable to rise from floor; grade 6, unable to climb stairs; grade 7, unable to rise from a chair; grade 8, unable to walk without assistance; and grade 9, unable to eat, drink or sit without assistance. Disease progression was considered as "slow" when there was only a grade change during a 5-year period, "moderate" when there was a two-grade change during the same period; "rapid" when there was a three-grade change or more. Muscle atrophy or hypertrophy, scoliosis, and contractures were also noted.

Among other routine blood tests, plasma CK levels were measured in all patients. Electromyography (EMG) was performed on most of them. Routine cardiological assessment including electrocardiogram (ECG) and echocardiogram and a respiratory

evaluation with spirometric investigation were performed at the time of diagnosis and regularly during follow-up.

The Fisher's exact test and Wilcoxon rank-sum test were used to compare genetic and protein data with clinical findings.

### Histopathological, IHC, and WB Analysis

In all probands muscle biopsy was taken from the brachial biceps or quadriceps muscle, after written informed consent. According to standard techniques, the muscle sections were analyzed by morphological study and IHC analysis. Dystrophin IHC was performed as described [Nicholson et al., 1989] using three monoclonal antibodies against the mid-rod domain, $NH_2$, and COOH epitopes (Novocastra, Newcastle upon Tyne, UK). IHC analysis with α-SG, β-SG, γ-SG, δ-SG, dysferlin (Novocastra), α-dystroglycan (α-DG) (Upstate Biotechnology, Lake Placid, NY), caveolin-3 (Transduction Laboratories, Lexington, KY), and telethonin monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) was performed as described [Prelle et al., 1998; Minetti et al., 1998; Valle et al., 1997].

The protein defects showed by IHC analysis were hence confirmed by WB analysis, using the same monoclonal antibodies. This technique was also used for the calpain-3 screening, using the Novocastra monoclonal antibody Calp 3d/2C4 [Anderson et al., 1998]. Band quantification on scanned images of WB membranes and gels was achieved using the software ImageJ (NIH; http://rsb.info.nih.gov). To take into account total protein load, band intensity was normalized to the densitometric value of residual myosin heavy chain stained by Coomassie blue. Quantitative values were expressed as the percentage of the mean densitometric data of two normal healthy muscle samples run on the same gel. Each sample was run in two different gels. Interexperimental variability was not significant for the following proteins both in patients and controls: dysferlin (patient samples n = 32, controls = 50); each sarcoglycan (disease sample = 20; controls = 48), and calpain 3 (patient samples = 72, controls = 68).

In eight LGMD2A and three LGMD2B patients only a semiquantitative analysis could be performed. These data are expressed as partial deficiency (PD; Supplementary Table S1; available online at http://www.interscience.wiley.com/jpages/1059-7794/suppmat).

### Molecular Analysis

According to the results of protein screenings, we have performed molecular analysis of the genes implicated in autosomal recessive and dominant LGMD: *MYOT* (encoding for myotilin), *LMNA* (lamin A/C), *CAV3* (caveolin-3), *CAPN3* (calpain-3), *DYSF* (dysferlin), *SGCA* (α-sarcoglycan), *SGCB* (β-sarcoglycan), *SGCG* (γ-sarcoglycan), *SGCD* (δ-sarcoglycan), and *FKRP* (Fukutin-related protein). DNA was extracted from peripheral blood samples obtained after written informed consent according to standard procedures. The exons and the adjacent intron regions were amplified by PCR using the conditions and genomic primers previously published, as follows: *MYOT* [Hauser et al., 2000], *LMNA* [Bonne et al., 2000], *CAV3* [Minetti et al., 1998], *CAPN3* [Richard et al., 1995], *DYSF* [Aoki et al., 2001; Cagliani et al., 2003a], *SCGA*, *SCGG*, *GSCG*, *DSCG* (as described in www.dmd.nd), and *FKRP* [Brockington et al., 2001]. PCR products were analyzed by direct sequencing (ABI Prism 3100 Genetic Analyzer, Applied Biosystems, Foster City, CA). Mutations were named according to the Leiden Muscular Dystrophy database (www.dmd.nl). Novel mutations were confirmed by PCR RFLP analysis, if they created or abolished naturally occurring restriction sites. The pathogenic nature of new mutations was confirmed by screening of 160 control Caucasian healthy subjects. The parental origin of each mutation was assessed trough analysis of parental genomic DNA, when available. In some patients, isolation of mRNA from muscle tissue, RT-PCR, and amplification of mRNA were performed.

GenBank reference sequences: CAV3: NM_000070.2; CAPN3: NM_033337.1;DYSF: NM_003494.2; SGCA: NM_000023.1; SGCB: NM_000232.3; SGCG NM_000231.1; SGCD: NM_000337.4; and FKRP: NM_024301.2. For cDNA numbering, +1 corresponds to the A of the ATG translation initiation codon in the reference sequence.

## RESULTS

A total of 181 LGMD patients from 155 families were selected. They presented a large clinical variability with respect to age of onset, patterns of skeletal muscle involvement, heart damage, and rate of progression. The male to female ratio was 1.03. The age of disease onset ranged from 1 to 55 years. Consanguinity was reported in nine families (12 patients). Anesthesia-induced rhabdomyolysis and malignant hyperthermia events were not observed.

According to the IHC and/or WB analysis, we identified 72 probands with a variable degree of calpain-3 deficiency, 31 with a dysferlinopathy, 32 with sarcoglycanopathies, two with caveolin-3 deficiency, four patients with abnormal α-dystroglycan glycosylation pattern, while 16 probands affected by a clinical phenotype of LGMD, with elevated serum CK levels and a dystrophic muscle biopsy pattern, showed normal expression at IHC and WB analysis of all investigated muscle proteins, including dystrophin, sarcoglycans, dysferlin, caveolin-3, telethonin, calpain-3, and merosin.

The mutation finding rate was 93% for dysferlin deficiency, 87% for any SG gene in patients with sarcoglycanopathies, while only 61% of probands with defects of calpain-3 carried mutations of the CAPN3 gene (Table 1).

### Autosomal Dominant LGMD

Only 10 patients presented an autosomal dominant pattern of disease transmission. All of them belong to two families affected by caveolinopathy due to two heterozygous mutations in the *CAV3* gene. The larger family has been previously reported [Cagliani et al., 2003b]; the second family showed a heterozygous p.Ala46Thr substitution: the proband was a 22-year-old man affected with mild lower and upper limb girdle muscle weakness with calf hypertrophy and dystrophic features at the muscle biopsy. The proband's mother was also affected with lower limb myalgias and had persistently increased CK values. All patients presented a mild disease progression and none of them lost the ability to walk after a mean follow-up period of 12.2 years (range, 3–35 years).

TABLE 1. **Correlation Between Protein Expression at IHC and/or WB Analysis and Probability to Identify Mutations in a Specific Gene**

| | Number of probands with | | |
|---|---|---|---|
| Protein | Protein defect (WB and/or IHC) | Pathogenic mutations | Yield (%) |
| Caveolin-3 | 2 (+ 8 family members) | 2 | 100 |
| Dysferlin | 31 (+ 6 siblings) | 29 | 93.5 |
| Sarcoglycans | 32 (+ 4 siblings) | 28 | 87.5 |
| Calpain-3 | 72 (+ 6 siblings) | 44 | 61.1 |

## Autosomal Recessive LGMD

***LGMD2A.*** A total of 77 patients, belonging to 71 families, had a variable degree of isolated calpain-3 deficiency (only one patient presented associated sarcoglycan complex and dystrophin deficiencies), therefore identifying calpain-3 deficiency as the most frequent protein alteration in our sample, being present in the 49.7% of our probands (77/155).

As previously observed [Saenz et al., 2005], calpain-3 deficiency was not always associated with mutations in the specific gene: in our sample we identified *CAPN3* mutations in 61% of screened probands with variable defects of calpain-3 protein amount, while the remaining patients showed a wild-type gene sequence. We also analyzed the *CAPN3* gene in all patients without a protein defect at the IHC and WB: only one patient with normal calpain-3 expression showed a heterozygous mutation in the *CAPN3* gene (Arg490Trp). Furthermore, a patient with calpain-3 deficiency showed mutations in the *FKRP* gene.

Patients with total absence or severe reduction of the 94 kD band at the WB analysis had higher probability to carry mutations than patients with partial defects. Genetic analysis, performed by direct sequencing of all 24 *CAPN3* exons, the exon–intron boundaries, the promoter, and the 3′UTR regions, showed mutations in 44 probands (Supplementary Table S1). *CAPN3* mutations were distributed along the entire length of the gene, although almost 60% of them fell within exons 4, 5, 10, and 11 (Fig. 1). Most of the *CAPN3* mutations represented private variants. The 550delA mutation had an allelic frequency of 8.8%. A total of 30 probands carried mutations in homozygosis or in compound heterozygosis, while 14 showed only one mutant allele. In two patients belonging to this second group, major genomic deletions were excluded by RT-PCR from muscle cDNA. In two probands, two different single-nucleotide changes were identified within the *CAPN3* promoter regions: the functional meaning of these changes is under evaluation.

All LGMD2A patients were affected with a predominant involvement of shoulder and pelvic girdle without distal muscle impairment. The disease showed a high clinical variability. The mean age of disease onset was 17.9 years (range, 2–55 years;standard deviation [SD] 12.05 years); 34 patients showed the first clinical sign before or at the age of 20 years and nine from 21 to 40 years; in four the disease started after the age of 40 years. Extensive clinical follow-up was available in 36 out of 46 (74%) patients. About 20% of patients were wheelchair bound, with a mean period from disease onset of 17.8 years (range, 4–29 years). Calf hypertrophy was rare and there was no mental retardation. No sex differences were evident in either age at onset or progression.

The mean CK level in these patients was 19-fold above the upper limit of normal (range, 2-fold to 110-fold). The EMG (available in 24/46 patients) showed a myopathic pattern in all probands except four cases, in which isolated or associated neurogenic signs were found. Among 42 patients who underwent to a complete heart assessment, none showed significant signs of cardiopathy. The spirometry (done in 29 patients) demonstrated normal respiratory function in 24 cases and a restrictive pattern in five patients.

In attempt to draw correlations between genotype and clinical severity, we compared type of mutations (truncating/truncating, truncating/missense, and missense/missense) and age of disease onset: generally null mutations resulted in earlier age of onset than missense mutations. In fact, patients carrying two null mutations had quite homogeneous and early age at onset of muscular symptoms (mean 12.2 ± SD 4.6 years), while patients with missense mutations (both in homozygosis or double heterozygosis) showed a later mean age of onset (16.8 ± SD 11.0 years) and also a wider range (2–45 years). However, the difference was not statistically significant.

***LGMD2B.*** Dysferlin deficiency was found in 37 mostly sporadic patients. The typical IHC pattern was characterized by absent or decreased sarcolemmal staining. Quantitative data of the WB analysis were available in 29 out of 35 patients. A total of 16 patients showed no detectable dysferlin band at WB analysis, while in the others the protein was variably reduced in amount. A double defect of both calpain-3 and dysferlin was observed in 11 patients. All patients carried mutation in the *DYSF* gene.

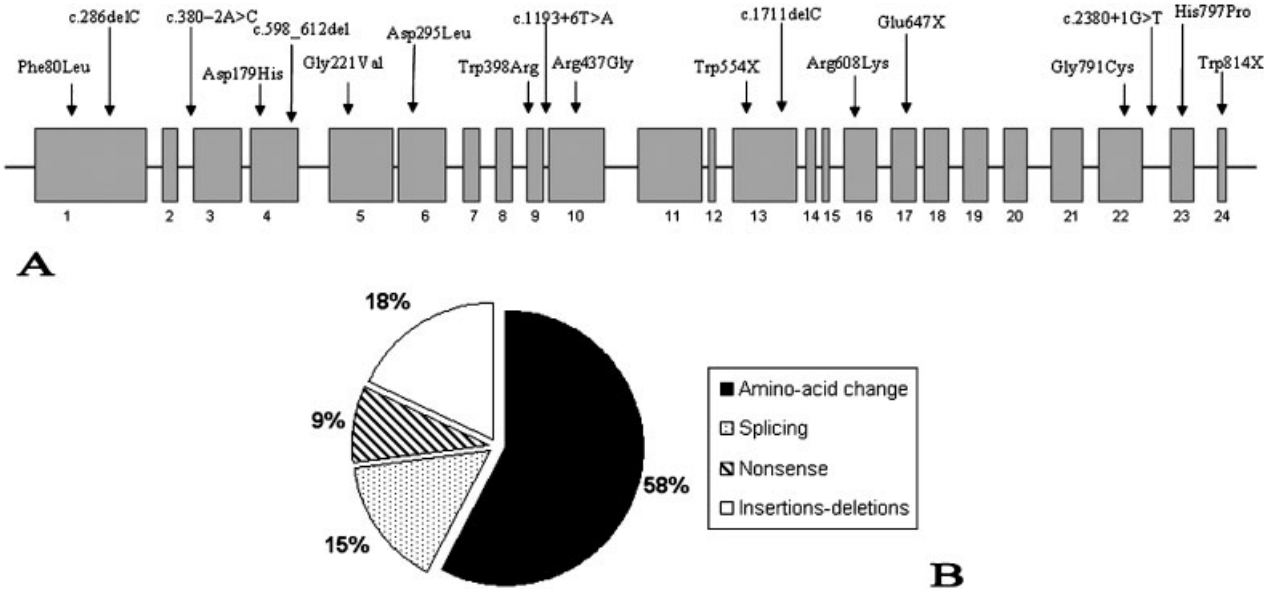


FIGURE 1. **Calpain-3 genetics. Molecular findings in LGMD2A patients. A: Schematic representation of intron/exon organization of *CAPN3* gene and distribution of new mutations along the gene. B: Relative frequency of the entire set of *CAPN3* mutations in our patients.**

A remarkable genetic heterogeneity was observed since 32 different mutations were identified in 29 probands: 13 mutations were in homozygosis, 10 in compound heterozygosis; in six patients only one mutant allele was identified, despite the fact that all coding and promoter regions were examined. The mutations were widely distributed in the coding sequence of the gene (Fig. 2A). Truncating mutations were the most frequent finding (21/32 = 65%). The c.2875C>T (p.Arg959Trp) mutation displayed a remarkable frequency, accounting for 13.8% of mutated alleles.

Most patients had a typical LGMD clinical phenotype, but seven showed both proximal and distal muscle weakness from the onset. No specific molecular data correlated with this feature.

The mean age of onset was 24.4 years (±SD 9.7 years), with a wide range (from 12 to 50 years). Out of 33 (52%) patients, 17 had a severe clinical phenotype with onset before or at the age of 20 years, 14 out of 33 (42%) experienced the first muscular symptoms between 21 and 40 years; and 2 out of 33 (7%) showed milder clinical features (onset after 40 years); three hyperCKemic patients were asymptomatic at the time of diagnosis (9%). The pace of symptoms evolution was variable. The 36% of patients had a moderate progression, while the 32% of them showed respectively a rapid and a slow disease evolution, 31 patients were evaluated for a mean follow-up time of 15 years (range, 2–41 years): seven patients were wheelchair-bound after a mean time of 21 years from disease onset (range, 5–40 years). Serum CK levels appeared extremely high (from 400 U/L to 25,000 U/L).

A total of 22 LGMD2B patients underwent to complete cardiac evaluation, which demonstrated a normal heart function in 15 patients, cardiac rhythm changes in three cases, and left ventricular hypertrophy in four subjects.

As far as molecular correlates are concerned, there was a significant association between mutation type and dysferlin amount at the WB analysis (Fisher's exact test for count data, $P = 3.4 \times 10^{-7}$) (Fig. 2B–D). Homozygous or double heterozygous truncating mutations were associated with a total absence of

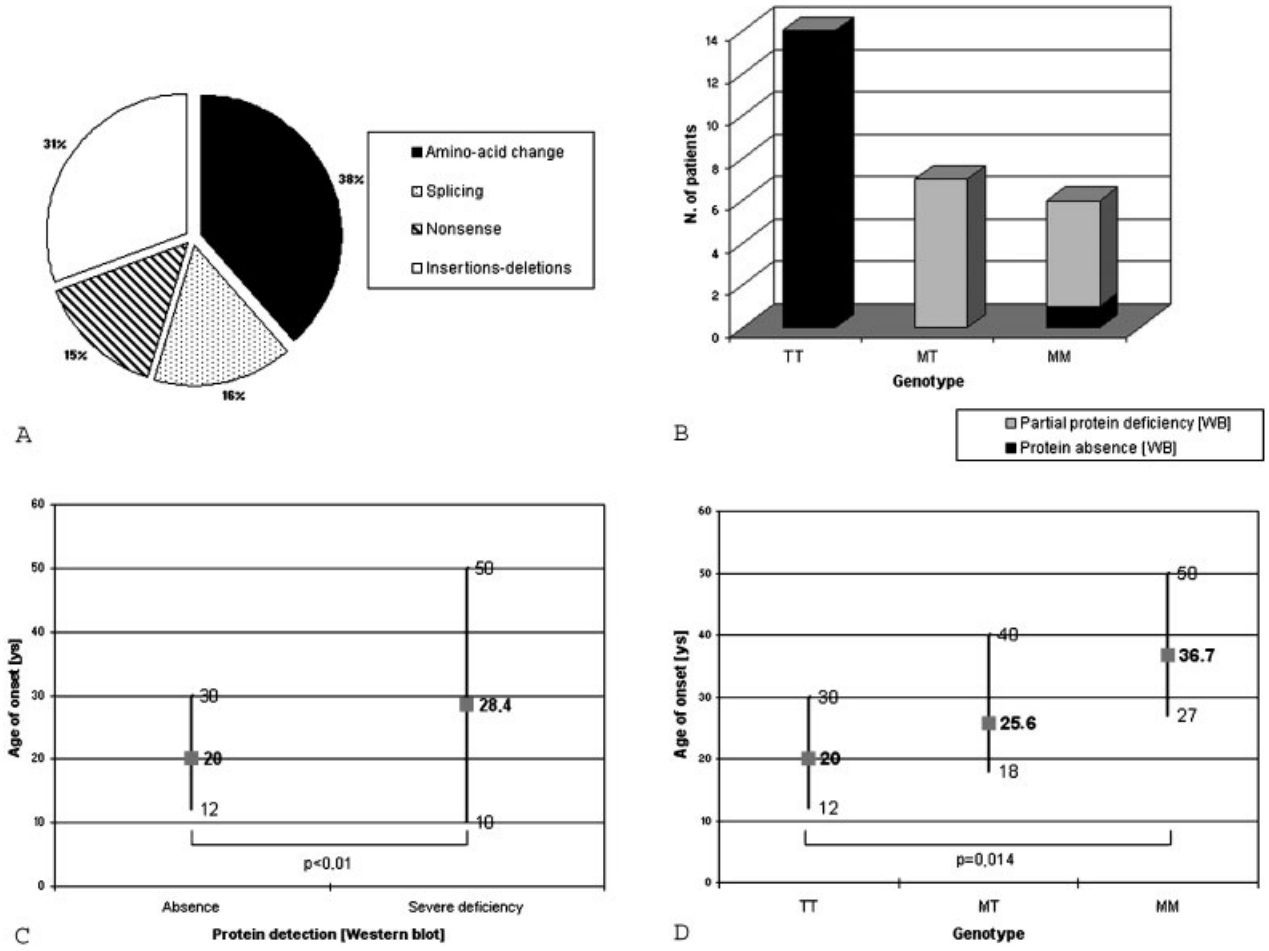


FIGURE 2. **Molecular analysis and genotype–phenotype–protein correlation among LGMD2B patients. A: Relative frequency of different types of *DYSF* gene mutations. B: Correlation between genotype (T = truncating mutation, M = missense mutations) and quantitative protein expression at WB analysis. Truncating mutations were always associated to a complete protein deficiency, while the presence of at least one missense mutation led to a milder deficit. C: Correlation between nature of mutations and phenotype (age of onset in years). Each line shows the range of age of onset for each group (the highest and the lowest age at disease onset are given in the upper and lower parts of represented values); in bold the mean age of onset is expressed in years. The Wilcoxon rank-sum test comparison between the homogenous double truncating and double missense groups is also shown (bracket). D: Correlation between quantitative protein expression at WB and age of onset (years). Each line shows the range of age of onset for each group; in bold the mean age of onset. The bracket shows the result of the Wilcoxon rank-sum test comparing patients with absent protein with those with mild protein defects.**

muscle dysferlin, as expected. On the contrary, the presence of at least one allele carrying a missense substitution resulted in partial protein residue (except in one case).

Age of disease onset differed in relation with nature of mutations and residual protein amount and the correlation was statistically significant in both conditions. Indeed, patients carrying two truncating mutations showed first muscular symptoms earlier in life than subjects harboring double missense substitutions (20 ± 5.1 years vs. 36.7 years ± 11.1 years; Wilcoxon rank-sum test: P = 0.0037). Presence of a compound heterozygosis for missense and truncating mutations led to an intermediate age of disease onset (mean 25.7 ± 7.6 years). Patients with dysferlin absence had an earlier mean age of onset when compared to those with partial deficiency (20.2 ± SD 5.2 years vs. 28.4 ± SD 11.2 years; Mann-Whitney test: P = 0.014).

***LGMD 2C-D-E-F (sarcoglycanopathies).*** A total of 38 patients (belonging to 32 families) showed deficiency of one or more components of sarcoglycan-complex at IHC and WB analysis. We identified mutations in both alleles of one sarcoglycan gene in 28 probands (20 in homozygous and eight in double heterozygous), while no mutations were found in four of them. In these nonmutated probands, deletions of the dystrophin gene were previously excluded, as well as major changes in dystrophin expression. Furthermore mutations in the FKRP gene were also excluded.

In detail, we identified 15 LGMD2D patients (11 singleton cases and two siblings), nine LGMD2C (with three siblings), eight LGMD2E, and one LGMD2F patients. Overall, 24 mutations were identified, 10 of which were new (Supplementary Table S1). Interestingly, all nonmutated patients showed a milder reduction of the SG complex at IHC and WB compared to the genetically-defined patients. Protein defects did not reliably predict genotype, with the exception of γ-SG deficiency, which was more closely associated with *SGCG* gene mutations.

As a group, the sarcoglycanopathies showed a more severe clinical phenotype than other LGMDs. Among them, the LGMD2E patients had earlier disease onset, higher CK levels, and more frequent cardiac involvement. In fact, the mean age at onset was 6.4 years (SD 6.4 years; range 1–20) in LGMD2E, 11.0 years in LGMD2C (SD 6.7 years; range 2–20), and 12.5 years in LGMD2D (SD 9.2 years; range 2–30) (Fig. 3A).

Finally, also in sarcoglycanopathies, the degree of protein deficiency seemed to correlate with phenotype, as total absence of any sarcoglycan (15/25 patients) was associated with earlier mean age at disease onset than partial deficiencies: 6.9 ± SD 5.6 years vs. 15.8 ± SD 9.2 years) (Wilcoxon rank-sum test: P = 0.006) (Fig. 3B).

***LGMD2I.*** The FKRP gene was sequenced in all undiagnosed cases, including patients with calpain-3 and sarcoglycan deficiency and without mutations in the respective genes, resulting in 10 mutated patients. Their pattern of protein expression and their mutation data are listed in Supplementary Table S1. The common c.826C>A mutation had an allelic frequency of 40%.

LGMD2I patients showed a wide clinical spectrum ranging from infantile noncongenital forms to late-onset cases. Disease onset occurred from 5 to 54 years of age, with a mean age of 23.2 years (SD 17.8). The age of onset had a bimodal distribution, with a first group of patients showing an early age of onset (8 ± SD 3.7 years), and a second one with a later onset (38.4 ± SD 9.6 years) The adult forms had a milder course when compared to other LGMD cases: after a mean follow-up of 11 years, only one patient was wheelchair-bound and none experienced a rapid progression.

Serum CK levels were considerably elevated in all patients (2- to 45-fold). Among those who underwent a complete cardiac evaluation (7/10), evidence of cardiac involvement was found only in one patient, who also showed restrictive respiratory dysfunction.

As far as protein analysis is concerned, normal expression of all investigated proteins was noticed in four patients, while two of them showed respectively moderate calpain-3 and sarcoglycan complex deficiencies. Three infantile patients had an abnormal pattern of α-DG glycosylation, while the IHC pattern was normal in an adult subject.

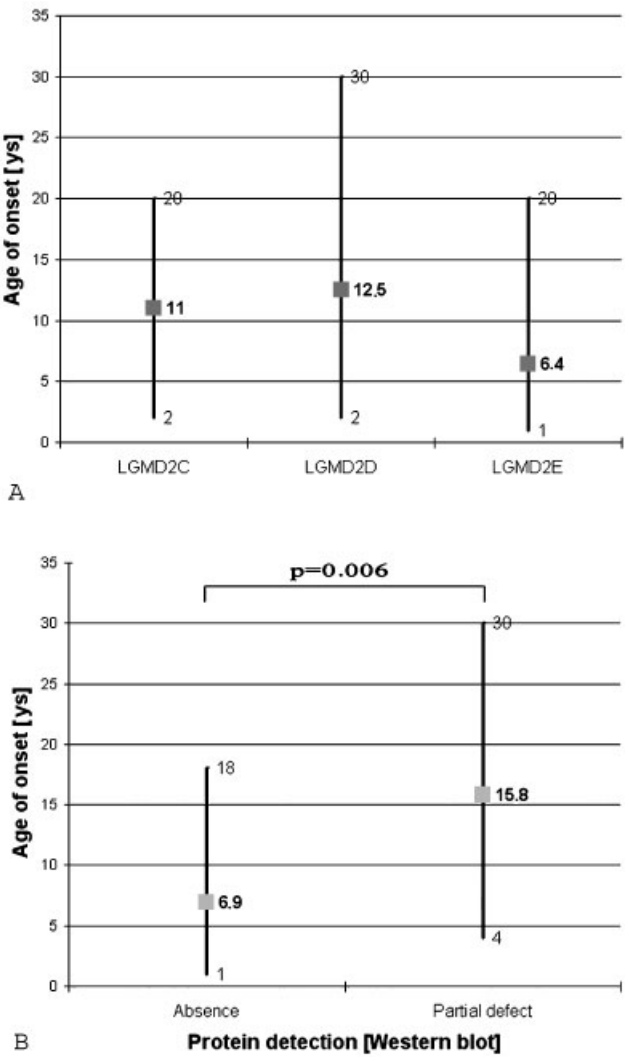


FIGURE 3. **Age at disease onset and molecular data in sarcoglycanopathies. A: Comparison between clinical presentation (age of onset) in different sarcoglycan forms. The LGMD2D form presents later onset compared to other sarcoglycanopathies. B: Correlation between quantitative protein expression at WB of any sarcoglycan and clinical presentation (age of onset in years). Statistical comparison is depicted by a bracket (see Results, Sarcoglycanopathies).**

## Unclassified LGMD2

Finally, in about 29% of our cohort, we did not identify mutations in any of the screened genes. These patients reported a family history suggestive of an autosomal recessive inheritance and fulfilled all inclusion criteria. The majority of them (26/41) showed a partial calpain-3 deficiency, four patients had sarcoglycan complex defects, only two patients showed dysferlin deficiency,

TABLE 2. Relative Frequency of Different LGMD Forms With Some Clinical Data in Our Cohort

| | OMIM | Gene symbol | Protein | Number of patients | Number of probands | % of Patients (% of probands) | Mean age at onset, years (range) | CPK levels (range) |
|---|---|---|---|---|---|---|---|---|
| LGMD1C | 607801 | CAV3 | Caveolin-3 | 10 | 2 | 5.5 (1.3) | 23 (3–49) | 2–8 × |
| LGMD2A | 253600 | CAPN3 | Calpain-3 | 48 | 44 | 26.5 (28.4) | 17.9 (2–55) | Normal–110 × |
| LGMD2B | 253601 | DYSF | Dysferlin | 35 | 29 | 19.3 (18.7) | 24.4 (10–54) | 3–125 × |
| LGMD2C | 253700 | SGCG | γ-Sarcoglycan | 9 | 7 | 5 (4.5) | 11 (2–20) | 4–90 × |
| LGMD2D | 608099 | SGCA | α-Sarcoglycan | 15 | 13 | 8.3 (8.4) | 12.4 (2–30) | 2–100 × |
| LGMD2E | 604286 | SGCB | β-Sarcoglycan | 8 | 7 | 4.4 (4.5) | 6.4 (1–20) | 2–110 × |
| LGMD2F | 601287 | SGCD | δ-Sarcoglycan | 1 | 1 | 0.5 (0.6) | 4 (4) | – |
| LGMD2I | 607155 | FKRP | Fukutin-related protein | 10 | 10 | 5.5 (6.4) | 23.2 (5–54) | 5–45 × |
| LGMD2 ? | | | ? | 45 | 42 | 24.9 (27.1) | 24.8 (1–53) | Normal–70 × |
| Total | | | | 181 | 155 | | (1–54) | Normal–125 × |

while in nine probands no specific protein deficiencies were detectable at IHC and WB analysis. All these patients were screened for mutations in the CAPN3 and FKRP genes without results.

## DISCUSSION

### Relative Proportion of LGMDs

LGMD diagnosis requires the integration of several contributions, including clinical findings, muscle biopsy evaluation, protein analysis, and finally molecular genetics. Unlike other neuromuscular diseases with distinctive clinical presentation, this process may be more difficult with LGMD patients, because of their genetic heterogeneity. Even if the majority of patients show a definable phenotype, exceptions to this general rule may be seen. Another factor contributing to heterogeneity may be occurrence of intrafamilial variability. Finally, different populations may have different distributions of the various LGMD forms. For these reasons, though the diagnosis may be suspected on clinical grounds, protein and genetic analysis are crucial to identify the different subtypes. Hopefully, protein data should address genetic investigations.

We performed a retrospective analysis of 181 LGMD patients followed for a mean of 13 years at multiple centers. Patients with a family history suggestive of an autosomal dominant inheritance resulted to have caveolin-3 deficiency. In LGMD1C, a positive protein analysis is predictive of positive genetic results. The issue is more complex for patients with an autosomal recessive inheritance: while the mutation finding rate in dysferlin deficiency is 93% in our sample, the isolated calpain-3 deficiency, which represents the most frequent protein alteration, leads to mutation detection in only 61% of cases.

Notwithstanding these diagnostic difficulties, genetically confirmed LGMD2A is the most represented LGMD type in our series, with a relative proportion of 28%. This finding is in agreement with data reported by other Italian studies [Fanin et al., 2005; Piluso et al., 2005] and by studies in other populations, which indicated a variable frequency, ranging from 26% in Japan [Chou et al., 1999], to 50% in Turkey [Dincer et al., 1997], to 80% in the Basque Country [Urtasun et al., 1998] and Russia [Pogoda et al., 2000].

In our cohort, the second more frequent form is LGMD2B. An early-generalized phenotype with both proximal and distal muscle weakness has been observed in six of our patients [Mahjneh et al., 2001; Nguyen et al., 2005]; without specific molecular correlates. The sarcoglycanopathies showed a relative proportion of 16.4% and LGMD2I comprises 6.5% of all LGMD forms. This latter frequency is much lower than that reported in Northern European populations, who present proportions ranging from 16% to 38% [Walter et al., 2004; Sveen et al., 2006]. Finally, in about 27% of cases no specific molecular diagnosis has been assigned, according to the data reported by other authors, even though the unclassified fraction might be different in different countries [Bonneman, 1999] (Table 2).

### Genetics

Based on molecular analysis, we may subdivide LGMD2 patients in three groups: 1) patients affected by a genetically determined LGMD2; 2) patients carrying only a single mutation in any of the LGMD2 genes; and 3) patients with LGMD clinical phenotype but no mutations in the known involved genes (with altered or normal expression of any specific protein).

In this study, we identified only one mutant allele in the specific gene in 23 LGMD2 probands, although all coding, promoter, 3′ and 5′UTR regions were examined: this finding was particularly frequent in patients with calpain-3 deficiency (14 probands in our cohort), but also five patients with dysferlin deficiency and four LGMD2I patients carried only one mutation in the *DYSF* and *FKRP* gene, respectively. Several criteria suggested inclusion of these patients within a given diagnostic category: obligate parental carriers of mutations were not affected, patients were symptomatic, and their muscle biopsy was dystrophic. Furthermore in most of these cases, IHC and WB analysis showed protein absence or severe deficiency. These criteria exclude instances of symptomatic heterozygosis, as those described for dysferlin deficiency [Fanin et al., 2006]. Incomplete mutation detection is the likely explanation of these cases [Richard et al., 1999; Fanin et al., 2005; Piluso et al., 2005]. Mutations lying in functional noncoding regions such as conserved regulatory elements or introns would have escaped to our screening procedure, as well as heterozygous macrodeletions, in patients lacking muscle tissue to explore cDNA integrity.

Another open genetic issue regards the cases with a specific protein defect at WB analysis, but without identified mutations in the corresponding gene. Two patients showed a dysferlin deficiency at IHC and WB, without mutations in *DYSF*. The question is still more significant considering the large number of apparently genotypically wild-type calpain-3–deficient subjects. In these patients, we excluded the presence of dysferlin WB abnormalities and screened for mutations in *FKRP* gene, which eventually led to the identification of one LGMD2I patient. As in single heterozygous patients, limitations of the techniques used for genetic screening can not be excluded, but we must also consider other gene defects with secondary calpain-3 deficiency (such as titin) [Udd et al., 2005] and the protein intrinsic instability [Haslbeck et al., 2005].

Finally, there is a group of patients characterized by a clinical phenotype of LGMD, a familial history suggestive for an autosomal recessive inheritance, a dystrophic morphological pattern at muscle biopsy, but normal expression of all examined proteins (dystrophin, calpain-3, dysferlin, sarcoglycans, and telethonin). An extensive screening for the CAPN3 and FKRP genes allowed us to identify one LGMD2A and one LGMD2I patient. It is therefore likely that other LGMD genes will have to be discovered.

### Genotype, Phenotype, and Protein Expression Correlations

Interestingly, LGMD2B patients differed in terms of mean age at disease onset depending upon the nature of their mutations and muscle protein expression. Double truncating mutations, leading to total absence of muscle dysferlin, result in earlier onset of muscle symptoms, compared to double missense mutations. This second group had a milder clinical phenotype and showed a variable degree of residual protein. Finally patients with a compound heterozygosis for missense and a frameshift mutation showed a more severe muscle dysferlin deficiency and an intermediate clinical phenotype. This correlation among genotype, age of onset, and protein expression, suggests that the defective membrane repair mechanism in response to sarcolemmal injuries is sensitive to the degree of dysferlin deficiency. Indeed a link between dysferlin amount and signs of muscle disease is also suggested by the observation that obligate carriers of DYSF mutations have a decreased expression of muscle dysferlin and increased values of serum CK [Fanin et al., 2006]. Therefore, at least in dysferlin deficiency, protein data may provide laboratory clues about disease severity.

In other LGMDs this association is weaker, although it is commonly observed that calpainopathic [Fanin et al., 2007] and sarcoglycanopathic patients with null mutations have a predictable more uniform and severe phenotype than patients with missense mutations and variable levels of protein expression. In this study, once divided in each separate diagnostic category, the relatively low number of patients within each disorder prevents a validation of this concept. It is also known that missense mutations are not functionally equivalent either in terms of enzymatic activity (for calpain-3) [Milic et al., 2007] or for assembling macromolecular complexes (for sarcoglycans), making prognosis a more difficult task. Therefore, larger databases, hopefully containing comparable observations, are needed to validate prognostic markers.

In conclusion, the molecular analysis remains the gold standard for the diagnosis of the different subtypes of LGMDs. Together with genetic analysis, an extensive collection of protein and clinical data should provide prognostic markers to predict disease severity and rate of progression.

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