Autosomal recessive limb-girdle muscular dystrophies diagnosed at Coimbra University Hospital

Distrofias Musculares das Cinturas autossómicas recessivas diagnosticadas nos Hospitais da Universidade de Coimbra

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Introduction

Limb-girdle muscular dystrophies (LGMDs) are a heterogeneous group of muscle diseases. Autosomal dominant (LGMD1) and recessive (LGMD2) forms are recognized, each one with several subtypes. In Portugal there are no studies reporting the relative distribution of the different subtypes of LGMD2.

Objective

To determine the subtypes of LGMD2 diagnosed and their relative distribution at the Neurology Department of the Coimbra University Hospital.

Material and Methods

The medical files of the patients with a diagnosis of LGMD2 were analysed and individual clinical, laboratory, pathologic and molecular data were recorded. The time frame of analysis was from 2000 to 2010.

Results

Forty-two patients from thirty-nine unrelated families were identified with a LGMD2 diagnosis. There were twenty-three female and nineteen male patients. Parental consanguinity was reported in eighteen patients (fifteen families). Their actual mean age is 44.6 years and the mean age of first symptoms was 23.2 years. The mean time from first symptoms to genetic diagnosis was 16.2 years. Twenty patients are wheelchair bound and seventeen can't raise the arms above the shoulder level. Three patients presented symptomatic dilated cardiomyopathy and twelve patients a restrictive respiratory syndrome, which was severe in five. The mean CK value was elevated in all LGMD2 subtypes. Immunohistochemistry suggested the specific diagnosis in twenty patients (LGMD2B: 11; LGMD2C-F: 9). Molecular studies performed in forty-one patients revealed 27 homozygous mutations, 11 compound heterozygous mutations and 3 heterozygous mutations. The LGMD2 subtypes diagnosed and the number of patients of each subtype was: LGMD2A: 5, LGMD2B: 16, LGMD2C-F: 9 (one patient without molecular study), LGMD2G: 1, LGMD2I: 7, LGMD2J: 1, LGMD2L: 3.

Conclusion

This retrospective analysis shows that most of the autosomal recessive LGMDs subtypes are represented in Portugal, being the LGMD2B subtype the most frequent. Rarer subtypes, like LGMD2G and J, were also found rare.

Key-words: Limb-girdle muscular dystrophies in Portugal; LGMD; autosomal recessive LGMD

Running title: AR LGMD at Coimbra University Hospital

Introdução

As Distrofias Musculares das Cinturas (DMC) constituem um grupo heterogéneo de doenças musculares. Existem as formas autossómicas dominantes (DMC1) e recessivas (DMC2), cada uma com vários subtipos. Em Portugal não há informação científica sobre a distribuição relativa dos diferentes subtipos de DMC2.

Objectivos

Avaliar os subtipos de DMC2 diagnosticados no Serviço de Neurologia dos Hospitais de Universidade de Coimbra e a sua distribuição relativa.

Material e Métodos

Análise dos processos clínicos dos doentes com o diagnóstico de DMC2 no período compreendido entre 2000 e 2010 e registo dos dados clínicos, laboratoriais, patológicos e moleculares individuais.

Resultados

Foram diagnosticados 42 doentes com DMC2, pertencendo a 39 famílias distintas e 23 eram do sexo feminino e 19 do sexo masculino. Consanguinidade parental foi identificada em 18 doentes, correspondendo a 15 famílias. A idade média actual é de 44.6 anos e a idade média dos primeiros sintomas de 23.2 anos. O tempo médio decorrido entre os primeiros sintomas e o diagnóstico molecular foi de 16.2 anos. Vinte doentes tinham perdido a marcha e dezassete não conseguiam elevar os braços acima do nível dos ombros. Três doentes apresentavam cardiomiopatia dilatada sintomática e sindroma respiratório restritivo foi diagnosticado em doze doentes, que era grave em cinco. O valor médio de CK estava elevado em todos os subtipos de DMC2. O estudo imunohistoquímico foi sugestivo do diagnóstico específico em vinte doentes (LGMD2B: 11; LGMD2C-F: 9) e o estudo molecular realizado em 41 doentes revelou mutações homozigóticas em 27 doentes, mutações em heterozigotia composta em 11 doentes e mutações em heterozigotia simples em 3 doentes. Os subtipos de DMC2 diagnosticados e o número de doentes por cada subtipo foram: DMC2A: 5, DMC2B: 16, DMC2C-F: 9, DMC2G: 1, DMC2I: 7, DMC2J: 1, DMC2L: 3.

Conclusão

Esta análise retrospectiva revela que a maioria dos diferentes subtipos de DMC2 estão presentes em Portugal, e o subtipo DMC2B foi o mais frequentemente diagnosticado. Os subtipos mais raros, como DMC2G e J, também foram diagnosticados, sendo o seu número muito reduzido.

Palavras-chave: Distrofias musculares das cinturas em Portugal; DMC; DMC autosómica recessiva

Título de cabeçalho: DMC AR nos Hospitais da Universidade de Coimbra

Introduction

Limb-girdle muscular dystrophies (LGMDs) are a heterogeneous group of inherited muscle disorders. Classified in two forms according to the mode of heredity[1], currently they comprise seven autosomal-dominant (LGMD1A to G subtypes) and fourteen autosomal-dominant subtypes (LGMD2A to N subtypes) and seventeen of them have their protein products identified. The LGMD1 is relatively rare and represents probably less than 10% of all LGMD cases.

LGMDs are characterized by muscle weakness and wasting of the scapular and pelvic girdle muscles with preservation of the facial muscles and high CK values. It is recognized extreme variability of the age of onset, the degree of muscle weakness, the pattern of muscular involvement and the level of disability and co-morbidities, like cardiac and respiratory involvement[2].

LGMDs have been the subject of numerous reviews and individual reports. Variable relative distribution of the different subtypes of the LGMD2 form have been presented, probably reflecting different ethnic backgrounds and geographic origins[3,4]. Some studies have found that the subtypes LGMD2A[5], 2C-F[6] and 2I[7] were the most common in each of the different countries where the studies were conducted, with small differences among them.

In Portugal, individual clinical cases[8,9] and small clinical series[10,11] about the individual LGMD2 subtypes have been published, but the relative distribution of the different subtypes of the LGMD2 has not been studied yet.

The Outpatient Neuromuscular Clinic of the Coimbra University Hospital is the reference center for the study of adult neuromuscular diseases in the central region of Portugal, a geographical area with a population of about 1.5 million people.

Here we describe the clinical, laboratory, pathologic and molecular data of a group of patients with LGMD2.

Material and Methods

Patient Population

The medical files of the Outpatient Neuromuscular Clinic of the Coimbra University Hospital were scrutinized to look for patients with a LGMD2 diagnosis. The time frame of the analysis was from 2000 to 2010. To be included in the study they had to have definite weakness on clinical examination primarily of the shoulder-girdle and pelvic muscles, a muscular biopsy showing dystrophic (or myopathic) features and a molecular study confirming the clinical and/or pathologic diagnosis of LGMD2. Patients with a diagnosis of dystrophinopathy or other neuromuscular disease confirmed by immunohistochemical or molecular genetic studies (FSHD, myotonic disorders, inflammatory muscle diseases and mitochondrial myopathy and glycogen or lipid storage myopathies) were excluded, as well as patients with weakness and wasting in the scapular and pelvic girdle muscles in which the etiologic investigation had been inconclusive. The LGMD2 subtypes are individually designated by the accepted nomenclature (1). LGMD2A indicates calpainopathy (*CAP-3* gene), 2B dysferlinopathy (*Dysf* gene), 2G telethoninopathy (*TCAP* gene), 2I α-dystroglycanopathy (*FKRP* gene), 2J titinopathy (*TTN* gene) and 2L anoctaminopathy (*ANO5* gene). In the text, the sarcoglycanopathies comprising the subtypes LGMD2C (γ-*sarcoglycan* gene), D (α-*sarcoglycan* gene), E (β-*sarcoglycan* gene) and F (δ-*sarcoglycan* gene) are collectively referred LGMD2C-F subtype and only when necessary each one is referred individually. The LGMD2 subtypes H, M and N are not mentioned in the text because they were not diagnosed in the time frame of analysis. Two brothers of patients with a LGMD2 diagnosis (LGMD2B and LGMD2A), without muscle biopsy but with a positive molecular study, were included in the analysis. Seven other patients were included, four with initial distal weakness later proven to be caused by pathogenic mutations in one of the LGMD2 genes and the other three patients, one of of gypsy descend with a suspected LGMD2C by immunohistochemistry (did not perform the appropriate molecular study) and the other two patients of the LGMD2C-E subtype confirmed by molecular studies requested by our centre, had muscle biopsy performed elsewhere and only the final impression of the pathological data was available.

Fifty-eight patients with limb-girdle muscular dystrophy diagnosis were identified. Sixteen had no definite diagnosis (unclassified LGMD). Forty-two patients with a definite LGMD2 diagnosis were included in the study, corresponding to 39 families.

Clinical Evaluation

The following data was recorded from the medical file of each patient: **1- historical features:** present age, gender, birthplace, race, ethnicity, consanguinity, family history of neurological disorders, age of first symptoms, initial site of first symptoms (lower limbs - proximal or distal, upper limbs - proximal or distal), age of muscular biopsy, age of molecular genetics diagnosis, time of muscular biopsy and molecular genetic study from the age of first symptoms, concurrent medical diseases and current medication; **2- physical evaluation:** height, weight, vital signs, records of involvement of other organ systems, muscular hypertrophy; **3- functional tests:** walking (independent, with support, impossible), arising from a chair (independent, with

hand support, impossible), raising arms (normal, shoulder level, below shoulder level, impossible), cardiovascular and respiratory functions (electrocardiogram, echocardiogram and functional respiratory evaluation). The highest serum creatine kinase value present on the medical file of each patient was recorded.

Muscle Biopsy Evaluation

The reports of the muscle biopsies were evaluated by one of us (L.N.) who recorded and graded the most prominent features: variability of fibre diameter (normal; increased, if superior to 3%), internal nuclei (absent; increased), necrotic fibres (absent; present: rare or frequent), basophilic fibres (absent; present), predominance of fibre type, connective tissue (normal; increased: focal or generalized), fat infiltration (absent; present: focal or generalized) inflammatory infiltrates (absent; present - localization), vascular abnormalities and the presence of special histopathologic features (lobulated fibres, rimmed vacuoles).

The muscle biopsies were routinely processed accordingly to procedures already described (10), namely: the biopsy fragments were frozen in isopentane chilled in liquid nitrogen and kept at a -70ºC. The transverse and longitudinal cryostat sections were cut 8µ thick, and stained by histochemical (H/E, PAS, Red-oil and Trichrome Gomori) and histoenzimatic routine methods (NADH-TR, SDH, ATPase pH4.35 and pH9.4) and cut 4µ thick for immunohistochemistry study (IHC) with antibodies against dystrophin (*dys* 1, *dys* 2, *dys* 3), α, β, δ and γ sarcoglycans, dysferlin, merosin, α-dystroglican and emerin (all from Novocastra). The intensity of staining with each antibody was graded from zero (absent) to 3+ (normal expression). Control human skeletal muscle was included with patient material on each glass slide immunostained in the study. At the time of the muscle biopsy some antibodies were not commercially available and if clinically indicated these were later studied in the biopsy fragments kept frozen at -70ºC in the laboratory. This happened in four muscle biopsies from patients with the LGMD2B subtype, which were evaluated with antibodies anti-dysferlin after the molecular diagnosis and all the muscle biopsies from patients with the LGMD2I subtype were evaluated with antibodies anti α-dystroglican after the molecular diagnosis.

A total of 38 reports of muscle biopsies were available and evaluated (two patients clinically affected, one brother of a LGMD2A female patient and a sister of a LGMD2B female patient did not perform muscle biopsy). Two patients had a muscle biopsy performed elsewhere and only the final impression with the results of the IHC (two LGMD2C-F patients) was available.

Molecular Studies

The molecular genetics studies were requested directed by the clinical examination and/or the results of muscle biopsy protein findings and were performed at the Molecular Genetics Unit of the Institute of Jacinto Magalhães, Porto.

1. gDNA analysis: Genomic DNA was extracted from peripheral blood by the salting-out method (12). Normal or M13-tailed primers used to amplify all the coding exons and directly flanking intronic sequences, were designed with aid of Primer Express (Applied Biosystems, Foster City, CA). Amplicons were purified using ExoSAP-IT (USB Corporation, Cleveland, OH) and sequenced with the respective normal or M13 universal primers, using the Big-DyeTM Terminator Cycle Sequencing Kit V1.1. The products were resolved on an ABI 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA). Mutation analysis was aided by SeqScape V2.5 software (Applied Biosystems, Foster City, CA) and Alamut V2.1 (Interactive Biosoftware, Rouen).
2. Transcript analysis: Total RNA was extracted from peripheral blood and/or muscle biopsies of patients and controls using TRIzol isolation reagent (Invitrogen, CA), and reverse transcribed using either Superscript One-Step RT-PCR with Platinum Taq (Invitrogen, CA) or the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City CA). Primers were designed according to case-specific interrogations. Amplicons resolved on 1% w/v agarose gels were eluted and sequenced in both directions, as described above.
3. Mutation characterization: Sequence variants in each gene were described according to the mutation nomenclature recommendations of the Human Gene Variation Society (HGVS) (13), using cDNA reference sequences filed under the following respective accession numbers: CAPN3 – NM_000070.2+BX499738.1; DYSF – M_001130978.1; MYOT - NM_006790.2; TCAP – NM_003673.3; ANO5 – NM_213599.2; FKRP – NM_024301.2+AC008622.6; SGCA – NM_000023.1 +L34355.1; SGCB – NM_000232.3+CN483961.1; SGCG – NM_000231.2; SGCD – NM_000337.4; FCMD - NM_139309.2; POMT1 - NM_007171.2; POMGNT1 - NM_017739.2.

Undocumented variants, for which pathogenicity could not be ascertained by transcript analysis or co-segregation studies, were evaluated by performing a population screen (100-150 anonymised control samples) using single-strand conformation analysis, high resolution melting-curve analysis or direct sequencing.

Results

A- Clinical Evaluation

1- Historical features (Table 1)

A total of 42 patients, representing 39 unrelated families were enrolled in the study. There were 23 (54.7%) female patients and 19 (45.2%) male patients, all of Caucasian race, with a Portuguese female of gypsy descent. Parental consanguinity was identified in 18 patients [(32.7%), (LGMD2A: 2, LGMD2B: 8, LGMD2C-E: 2, LGMD2I: 5, LGMD2J: 1)] corresponding to fifteen families. Family history for similar neuromuscular disorder was positive in four LGMD2 subtypes: two affected brothers in each of the subtypes LGMD2A, LGMD2B and LGMD2L, and two patients in the LGMD2I subtype were second-degree cousins.

The mean age of enrolment was 44.6 years and the mean age of first symptoms was 23.2 years. The mean age of first symptoms was below the age of twenty in 24 patients (57.1%), with 9 patients showing the first symptoms in the first decade, with 6 of these belonging to the LGMD2C-F subtype. Only five patients had their first symptoms at or after the age of 50. The mean age of first symptoms was lowest in the LGMD2C-F and higher in the LGMD2L subtypes. The mean age of molecular diagnosis was 38.5 years, with the LGMD2C-F and LGMD2L subtypes presenting the lowest and highest mean age, respectively, and reflecting the difference of onset of the disease of each subtype. The mean time from first symptoms to muscle biopsy and molecular diagnosis was 13 years and 16.2 years, respectively. Only one patient with the LGMD2G and another one with the LGMD2J subtypes were found, which are very rare outside the countries where they were first described (14,15) and the age of and the time to molecular diagnosis of these two subtypes were 50/30 years and 24/2 years, respectively. All patients, except two without weakness, reported the initial site of first motor symptoms in the legs, 36 proximally and 4 distally.

2- Physical Evaluation (Table 1)

No medical condition was identified, like endocrine or metabolic diseases that could explain or be related to the weakness. Fourteen patients presented calf hypertrophy (33.3%), distributed by the subtypes LGMD2A, LGMD2C-F (one of these patients also had macroglossia), LGMD2D, LGMD2I and LGMD2G.

3- Functional Tests (Table 2)

Twenty-one patients (50%) were able to walk without support, one with support and twenty were confined to a wheelchair. According to the LGMD2 subtypes, the LGMD2C-F subtype presented the highest percentage of patients wheelchair bound (77.7%), but the subtypes 2A, 2B and 2I (13 in a total of 28 patients) also presented a significant percentage of wheelchair bound patients (46.4%). The patients of the LGMD2G, J and L subtypes were able to walk without support. The ability to rise from a chair without hand support was preserved in 16 patients; three patients needed hand support and twenty-three patients were unable to rise from a chair. According to the different subtypes the LGMD2C-F (8 out of 9) and 2I (5 out of 7) subtypes were those with the highest percentage of patients with this motor disability.

The number of patients with ability of raising the arms above their head was 13, three patients from LGMD2L subtype and six from the LGMD2B subtype. Only one patient from each of the other subtypes was able to raise the arms above head (except the patient from LGMD2G subtype). Seventeen patients with the highest percentage of patients belonging to the LGMD2B and 2C-F subtypes were not able to raise the arms above head. Twelve patients (28.5%) were able to raise the arms to shoulder level.

From the historical data and the last three functional tests, it is clear the progressive nature of the disease, with progressive severity of the weakness with time in the lower limbs, and the upper limbs being involved later (or if present since the beginning of the disease not so severely affected as the lower limbs). The severity of weakness of the upper limbs and the resulted disability was less severe than that found in the lower limbs.

The patients from the LGMD2B, 2G, 2J and 2L subtypes had no signs or symptoms of cardiac or respiratory insufficiency, and this is in accordance with the experience of other centres. These medical complications were found in 12 patients from the LGMD2A, 2C-F and 2I subtypes. Combined symptomatic dilated cardiomyopathy and severe restrictive respiratory syndrome (needing intermittent ventilator support) was present in one patient of the LGMD2D subtype and in two patients of the LGMD2I subtype. Five patients of the LGMD2C-F subtype had a severe restrictive respiratory syndrome (LGMD2C: 2, 2D: 2, 2E: 1) with one patient of the LGMD2D subtype needing intermittent ventilator support. Four other patients (LGMD2A: 2 and LGMD2I: 2) had a restrictive respiratory syndrome of variable severity, with only one patient from the LGMD2A subtype needing intermittent ventilator support.

The individual CK values were elevated in 40 patients (it was normal in two patients, one from the LGMD2B subtype and the other from LGMD2C subtype) and the mean CK value was elevated (2866 UI/L). The highest individual CK value was recorded in a patient of the LGMD2B subtype (25 648 UI/L) and the highest mean value was also recorded in

Table 1. Patient population and clinical evaluation (historical features and physical evaluation)

	Sex F/M	Mean Age (years)			Mean age of molecular diagnosis (years)	Time to (years)		Calf hipertrophy
		Actual	1st symptoms	1st symptoms by decade		Biopsy	Molecular diagnosis	
All patients (n = 42)	23/19	44.6 ± 15.1 (21 - 75)	23.2 ± 16.4 (4 - 69)	1st - 9 / 2nd - 15 / 3rd - 10 / 4th - 1 / 5th - 2 / 6th – 3; 7th - 2	38.5 ± 15 (12 – 74) (n = 39)	13 ± 9.95 (2 – 32) (n = 40)	16.2 ± 11 (2 – 43) (n = 41)	14 (34.4%)
LGMD2A (n = 5)	3/2	44.2 ± 12.8 (31 - 62)	14.9 ± 14.9 (8 - 46)	1st - 1 / 2nd - 3 / 5th - 1	40.6 ± 14.2 (30 – 59)	17.7 ± 7.2 (11 – 24) (n = 4)	19.8 ± 11.5 (9 – 38)	1
LGMD2B (n = 16)	7/9	46.5 ± 17.2 (26 - 75)	28.3 ± 17.3 (15 - 69)	2nd - 8 / 3rd - 4 / 4th - 1 / 6th - 1 / 7th - 2	41.4 ± 19 (21 – 74)	8,6 ± 7.9 (3 - 32) (n = 15)	13.1 ± 11.6 (3 – 43)	
LGMD2C-F (n = 9)	7/2	35.5 ± 10 (21 - 49)	11.6 ± 8.15 (4 - 28)	1st - 6 / 2nd - 2 / 3rd – 1	29.5 ± 111.7 (12 - 46) (n = 8)	16.3 ± 9.2 (4 - 30)	18.5 ± 8.4 (5 – 29)	5 (one with macroglossia)
LGMD2I (n = 7)	5/2	43.4 ± 9.57 (34 - 61)	18.1 ± 10.4 (6 - 30)	1st - 2 / 2nd - 2 / 3rd - 3	37.5 ± 9.53 (29 - 56)	15.1 ± 10.7 (2 – 27)	18.8 ± 10.7 (4 - 30)	7
LGMD2G (n = 1)	1/0	50	20	3rd - 1	50	30	30	1
LGMD2J (n = 1)	0/1	25	22	3rd - 1	24	2	2	
LGMD2L (n = 3)	0/3	69 ± 5.6 (65 - 70)	50 ± 7 (45 – 55)	5th - 1 / 6th - 2	68 ± 3.6 (64 - 71)	13.3 ± 9.45 (6 - 24)	17 ± 8 (9 - 17)	

LGMD: Limb-girdle muscular dystrophy; n: number of patients; F: female: M: male; 1st: first; 2nd: second; 3rd: third; 4th: fourth; 5th: fifth; 6th: sixth; 7th: seventh; ±: standard deviation

Table 2. Clinical evaluation: functional tests

	Walking			Rising from a chair			Raising Arms			Cardiovascular Respiratory Systems		CK (UI/L)
	Independent	With support	Impossible	Without support	With support	Impossible	Above head	Shoulder level	Below shoulder level			
All patients (n = 42)	21 50%)	1 (2.3%)	20 (47.6%)	16 (38%)	3 (7.1%)	23 (54.7%)	13 (30.9%)	12 (28.5%)	17 (40.4%)	3 (7.1%)	9 (21.4%)	2866 ± 4869 (n = 38)
LGMD2A (n = 5)	2	- -	3	2	- -	3	1	3	1		BiPAP (n = 1) SRS (n = 1)	941 ± 570.5 (279 - 1859)
LGMD2B (n = 16)	9	1	6	9	1	6	6	3	7	- -	- -	4975 ± 6520 (37 - 25648)
LGMD2C-F (n = 9)	2	- -	7	1	- -	8	1	2	6	DCM + BiPAP (n = 1)	BiPAP (n = 1) SRS (n = 4)	1724 ± 2500 (265 - 7376)
LGMD2I (n = 7)	3	- -	4	2	- -	5	1	4	2	DCM + BiPAP (n = 2)	SRS (n = 2)	1383.5 ± 875.6 (500 - 3045)
LGMD2G (n = 1)	1	- -	- -	- -	- -	1	- -	- -	1	- -	- -	476
LGMD2J (n = 1)	1	- -	- -	1	- -	- -	1	- -	- -	- -	- -	639
LGMD2L (n = 3)	3	- -	- -	1	2		3	- -	- -	- -	- -	3793 ± 1081 (2696 - 4858)

LGMD: Limb-girdle muscular dystrophy; n= number of patients; DCM: dilated cardyomyopathy; SRS: severe respiratory syndrome; BiPAP: bilevel positive airway pressure; ±: standard deviation

the LGMD2B subtype (4975 UI/L) and the lowest mean CK value was recorded in the LGMD2A subtype (941 UI/L).

B- Histopathology and Immunophenotypes

Reports of thirty-eight muscle biopsies were analysed. The majority of the muscles biopsies were dystrophic. One of the four muscle biopsies from patients of the LGMD2A subtype was considered myopathic and the muscle biopsies from patients of the subtypes 2J and 2L were also myopathic, without increased connective tissue or fat infiltration.

Before the antibodies anti-dysferlin became available, muscle biopsy from one patient of the LGMD2B subtype showed histopathologic findings that were suggestive of Inclusion Body Myositis, another of metabolic disease and one was considered normal. The muscle biopsies from patients of the LGMD2I subtype had no distinctive morphologic features and all of them were dystrophic. The muscle biopsy from the patient with the LGMD2G subtype presented lobulated fibres and rimmed vacuoles together with a muscular dystrophic pattern[9]. Lobulated fibers were also present in muscle biopsy from a patient of the LGMD2A subtype. Endomysial focal inflammatory infiltrate was found in the muscle biopsies of the two brothers with the LGMD2L subtype. The muscle biopsies from patients of the LGMD2B subtype had several distinctive histopathologic features, including frequent necrotic fibres (n=8) with four of these presenting focal inflammatory infiltrates and rimmed vacuoles. The presence of these three special histopathology features in the same muscle specimen was identified in two muscle biopsies. Frequent basophilic fibres (n=6) were identified together with necrotic fibres in five cases, and three of these also had focal inflammatory infiltrates.

In LGMD2B immunohistochemistry with anti-dysferlin antibodies revealed irregular immunostaining in two cases and absence in the others. In the LGMD2C-F subtypes, an abnormal imumunostaining of the sarcoglycans was present in all of them. In two muscle biopsies, all the sarcoglycans were absent (LGMD2D and LGMD2E). One muscle biopsy showed the absence of γ-sarcoglycan accompanied the reduced immunostaining of the other three sarcoglycans (LGMD2C), and in the other six muscle biopsies only one sarcoglycan was absent, with normal immunostaining of the other three. The diagnosis of the LGMD2I subtype was done by molecular study in all patients. The antibodies against α-dystroglican only became available after the molecular diagnosis, and when applied to the muscle specimens, the results of IHC were in accordance with the molecular ones. In our centre antibodies against telethonin, titin and Anoctamin 5, as well as Western blot analysis, are not available yet.

The diagnosis of the individual LGMD2 subtypes was possible by immunohistochemistry in twenty patients (47.6%) (LGMD2B: 11, LGMD2C-E: 9). All the other LGMD2 subtypes were diagnosed and confirmed by molecular studies (except one patient of the LGMD2C-F subtype).

C- Genotypes (Table 3)

Forty-one patients had an informative genetic test [the gipsy descent patient did not perform molecular study (suspected LGMD2C by IHC)]. Twenty-seven patients had a homozygous mutation. Of these, seven were frameshift mutations (LGMD2B: 3, LGMD2C: 1; LGMD2J: 1; LGMD2L: 2), seventeen were missense (LGMD2B: 6, LGMD2C: 1, LGMD2D: 2, LGMD2E: 1, LGMD2I: 7), one was a nonsense mutation (LGMD2G), another one was an aberrant splicing (LGMD2L) and the last one a deletion (LGMD2B). Eleven patients had compound heterozigoty (LGMD2A: 3, LGMD2B: 5, LGMD2C: 1, LGMD2D: 2). Mutations in heterozigoty were found in the LGMD2A (n=2) and LGMD2B (n=1) subtypes, all of the missense type.

The mutations in the *CAP-3* gene were found in exons 1 (n=3), 5, 9, 11, 22, in the *DYSF* gene (n=21) in exons 53 (n=4), 12 (n=4), 49 (n=4), 48, 18, 39, 29, 52, 15, 37, 7 and 50. All the missence mutations in the FKRP gene were located in exon 4, with only one with a different exon location of the mutation. The mutations in the sarcoglycans genes (n=11) were found in exons 6 (LGMD2C: 2, LGMD2D: 1, LGMD2E: 1), 3 (LGMD2D: 4) and 7 (LGMD2C: 2, LGMD2D: 1). The different types of sarcoglycanopathies diagnosed were divided in LGMD2C (n=3), LGMD2D (n=4) and LGMD2E (n=1) subtypes. The mutations in the *TCAP* and *TTN* genes were located in the exons 2 and 363, respectively and in the ANO5 gene the mutations were located in exons 18 and 5 (n=2).

In the present study, LGMD2B was the most common subtype diagnosed (38%), followed by sarcoglycanopathies as a group (21.4%), LGMD2I (16.6%) and LGMD2A (11.9%). The LGMD2G and 2J are very rare autosomal recessive LGMDs and ANO5 gene mutations only recently were found in LGMD2 patients (16), so the number of patients with this subtype might be higher than what it was found.

Discussion

Since the first time a genetically confirmed LGMD2 was presented at the Portuguese Society of Neurology[16], the number of LGMD2 subtypes increased significantly in the subsequent ten years. The gene defects discovered affect various sites throughout the muscle fibre, including the nuclear envelope, sarcomere, sarcoplasm and sarcolemma. The frequent clinical phenotype overlap among the different LGMD2 subtypes makes it difficult to come up with an immediate and accurate diagnosis of a specific LGMD2. Immunohistochemistry and Western blot analysis first and molecular studies later are, in most cases, necessary for a definite diagnosis.

The present study does not intend to give an estimate of the prevalence of the different subtypes of LGMD2 in the central region of Portugal, but the relative distribution of the different LGMD2 subtypes diagnosed in our centre. Recently, other hospitals from the central area of Portugal began to carry out independent investigation and diagnosis of patients with LGMD. While some of these patients have

Table 3. Molecular data

	Location of the mutation	Consequences at protein level		Location of the mutation	Consequences at protein level		Location of the mutation	Consequences at protein level		Location of the mutation	Consequences at protein level
LGMD2A			**LGMD2B**			**LGMD2C-F**			**LGMD2I**		
1	exon 1 heterozygous c.295T>A	p.Trp99Arg	6	- Intron 26 + exon 49 c.[2801+1G>A; c.5509G>A] - exon 53 c.5999G>A	(p.?) + p.Asp1837Asn p.Arg2000Gln	1	not available		1	exon 4 homozygous c.826C>A	p.leu276Ile
2	exon 1 c.60delA exon 22 c.2306G>A	p.Pro62fsX35 p.Arg769Gln	7	exon 49 homozygous c.5509G>A	p.Asp1837Asn	2	γ sarcoglycan gene exon 6 homozygous c.525delT	p.Phe175fsX20	2	exon 4 homozygous c.826C>A	p.leu276Ile
3	exon 5 heterozygous c.637C>T	p.His213Tyr	8	- exon 29 c.3115C>T - exon 52 c.5813_5821dup CAGCCAAGA	p.Arg1039Trp p.Thr1938_1940 Lysdup	3	α sarcoglycan gene exon 3 homozygous c.229C>T	p.Arg77Cys	3	exon 4 homozygous c.826C>A	p.leu276Ile
4	exon 9 c.1116-1G>A exon 11 c.1468C>T	p.Trp373ThrfsX59 + p.Trp373_Trp398del p.Arg490Trp	9	exon 12 c.1180_1180+7del exon 15 c.1379_1381del	p.Glu353_Leu429del p.Arg460del	4	α sarcoglycan gene exon 3 c.229C>T exon 7 c.850C>T	p.Arg77Cys p.Arg284Cys	4	exon 4 homozygous c.826C>A	p.leu276Ile
5	exon 1 c.60delA exon 22 c.2306G>A	p.Pro62fsX35 p.Arg769Gln	10	exon 49 homozygous c.5509G>A	p.Asp1837Asn	5	α sarcoglycan gene exon 3 homozygous c.229C>T	p.Arg77Cys	5	exon 4 homozygous c.826C>A	p.leu276Ile
LGMD2B			11	exon 53 homozygous c.5979dupA	p.Glu1994ArgfsX3	6	β sarcoglycan gene exon 6 homozygous c.323T>G	p.Leu108Arg	6	exon 4 homozygous c.545A>G	p.Tyr182Cys
1	exon 53 homozygous c.5979dupA	p.Glu1994ArgfsX3	12	exon 12 homozygous c.1180_1180+7del	p.Glu353_Leu429del	7	α sarcoglycan gene - exon 3 c.229C>T - exon 6 c.739G>A	p.Arg77Cys p.Val247Met	7	exon 4 homozygous c.826C>A	p.leu276Ile
2	exon 12 c.1180_1180+7del exon 53 c.5979dupA	p.Glu353_Leu429del p.Glu1994ArgfsX3	13	exon 37 homozygous c.4003G>A	p.Glu1335Lys	8	γ sarcoglycan gene exon 7 c.629A>G	p.His210Arg	**LGMD2J**		
3	exon 48 homozygous c.5429G>A	p.Gly178ValfsX17	14	exon 12 homozygous c.1168G>A	p.Asp390Lys	9	-γ sarcoglycan gene - exon 6 c.525delT - exon 7 c.629A>G	p.Phe175LeufsX20 p.His210Arg	1	exon 363 homozygous c.100185delA	p.Lys33395AsnfsXp9
4	exon 18 c.1620delA exon 39 c.4200dupC	p.Glu541SerfsX86 p.Ile1401HisfsX8	15	exon 7 homozygous c.757C>T	p.Arg253Trp	**LGMD2G**			**LGMD2L**		
5	exon 49 homozygous c.5509G>A	p.Asp1837Asn	16	exon 50 heterozygous c.5626G>A	p.Asp1876Asn	1	exon 2 homozygous c.157C>T	p.Gln53X	1	exon 18 homozygous c.2012ª>G	P.Tyr671_Val667delinsPhe
									2	exon 5 homozygous c.1991dupA	p.Asn64lysfsX15
									3	exon 5 homozygous c.1991dupA	p.Asn64lysfsX15

been referred to our centre for a definite diagnosis and included in the present study, others are diagnosed locally and not referred to us, so the number, form and subtype of LGMD are not of our knowledge. LGMD2B subtype was the most common diagnosis (38%), followed by the sarcoglycanopathies as a group (21.4%), LGMD2I (16.6%) and LGMD2A (11.9%) subtypes. Different authors have reported data about the relative distribution of LGMD2 subtypes. In Brazil, sarcoglycanopathies were found to be the most common, followed by dysferlinopathies[18]. In Italy, several authors found the LGMD2A subtype as the most common one, followed by dysferlinopathies[5,19]. In the USA, dysferlinopathies represented the largest subtype, followed, with similar data, by LGMD2I and LGMD2C-F[20]. In Denmark, LGMD2I was the most common subtype, with LGMD2B representing only 2% of the population studied[7]. The relative distribution of the different subtypes in our study, with small differences, might be considered similar to the above cited data. The only exception that deserves some comment is the relatively low number of patients with LGMD2A. It is possible that some cases might have been missed when the clinical phenotype was not a typical one. The use of Western bolt analysis could have helped the diagnosis of LGMD2A, but it is not available in our centre. It is important to remember the experience

reported by other centres with this technique showing that reduced amounts of calpain-3, sometimes even its absence it is not followed by the identification of pathogenic mutations in the *CAPN3* gene[21]. Another possible explanation is that LGMD2A, like sarcoglycanopathies, is more common in the paediatric than in adult population[22], which forms the majority of the population included in our study. In two cases of LGMD2A and one of LGMD2B subtypes only one mutation was found. Regarding the first one, this finding is not rare and some authors suggest that calpainopathy could be transmitted in an autosomal dominant mode. For dysferlinopathy, other reports have been published in which only one mutation was found and several explanations, related to the methodology of the molecular study and the nature and location of the mutation, were proposed[23,24].

Phenotype-genotype correlations have been attempted for all the LGMD2 subtypes. No general rules can be applied or a direct consequence can be drawn from the type and location in the gene of the mutation and the severity of clinical phenotype. The direct consequence of the defective protein over the integrity of the sarcolemma, the destabilization of the dystrophin-glycoprotein complex and the efficacy of the repair mechanisms of the injured sarcolemma are probably more important in determining the severity and progression of the muscular dystrophy than the mutation itself.

The mean times to muscle biopsy and molecular diagnosis were significantly high. The first one might be explained by insidious nature of most LGMDs and consequent delay of the patient and family in searching for medical care. Sometimes the delay in referring patients to specialized medical centre is a consequence of the rarity of the disease and the difficulty of the general practitioner in diagnosing a muscle disorder. Usually the molecular diagnosis is made after performing the muscle biopsy, so it is reasonable the longer mean time to reach a molecular diagnosis. Another reason for the delay in molecular diagnosis is that in some LGMDs the molecular diagnosis was not available at the time of the clinical evaluation and muscle biopsy, as it happened in LGMD2I and 2L subtypes.

It is important to have a specific molecular diagnosis, since it gives us the opportunity to inform the patients about the probable natural history of the disease, the probability of other members of the family to be clinically affected and to be more active in preventing and treating medical complications which in some subtypes are relatively common. It is impossible to perform molecular investigations for all the genes responsible for the known LGMD2 so, probably, in some patients the appropriate test is not requested. Since 1995, when mutations in the proteolytic enzyme calpain-3 were identified in patients with an autosomal recessive LGMD[25] pathogenic mutations in other genes and in different chromosomes have been progressively found in patients with previously undiagnosed LGMD2. It is reasonable to think that new genes coding for proteins not yet known to be responsible for LGMD2 will be found in the future and so, it is expected that the number of patients and the subtypes of LGMD2 will be higher in the future.

The diagnostic process of LGMDs needs to encompass many variables (clinical, laboratory, pathologic, etc) which difficult the elaboration of a diagnostic algorithm useful in the clinical practice. Each patient should be evaluated individually and the result of each test should be critically considered in accordance to clinical signs and symptoms. ■

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