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LIMB-GIRDLE MUSCULAR DYSTROPHY

An immunohistochemical diagnostic approach

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ABSTRACT - The limb-girdle muscle dystrophy (LGMD) represents a heterogeneous group of muscular diseases with dominant and recessive inheritance, individualized by gene mutation. A group of 56 patients, 32 males and 24 females, with suggestive LGMD diagnosis were submitted to clinical evaluation, serum muscle enzymes, electromyography, muscle biopsy, and the immunoidentification (ID) of sarcoglycans (SG) α, β, γ and δ, dysferlin and western blot for calpain-3. All the patients had normal ID for dystrophin (rod domain, carboxyl and amine terminal). The α-SG was normal in 42 patients, β-SG in 28, β-SG in 45, δ-SG in 32, dysferlin in 37 and calpain-3 in 9. There was a reduction in the α-SG in 7 patients, β-SG in 4, γ-SG in 2, and δ-SG in 8. There was deficiency of α-SG in 7 patients, β-SG in 6, γ-SG in 9, δ-SG in 5, dysferlin in 8, and calpain-3 in 5. The patients were grouped according the ID as sarcoglycans deficiency 18 cases, dysferlin deficiency 8 cases and calpain-3 deficiency 5 cases. Only the sarcoglycans deficiency group showed calf hypertrophy. The dysferlin deficiency group was more frequent in females and the onset was later than sarcoglycan and calpain-3 deficiency groups. The calpain-3 deficiency group occurred only in males and showed an earlier onset and weaker muscular strength.

KEY WORDS: limb-girdle muscular dystrophy, immunoidentification, sarcoglycans, dysferlin, calpain-3.

Distrofias musculares de cinturas: uma abordagem diagnóstica imuno-histoquímica

RESUMO - As distrofias musculares de cinturas (DMC) representam grupo heterogêneo de doenças musculares com heranças autossômicas dominante ou recessivas, caracterizadas geneticamente por mutações gênicas específicas. Cinqüenta e seis pacientes, 32 masculinos e 24 femininos, com diagnóstico sugestivo de DMC, foram submetidos a avaliação clínica, dosagem séricas das enzimas musculares, eletromiografia, biópsia muscular e imunoidentificação (ID) das proteínas sarcoglicanas (SG) α, β, γ e δ, disferlina e calpaína-3. A ID da distrofina (domínio rod e terminais carboxila e amino) era normal em todos os pacientes. Apresentaram ID normal para α-SG 42 casos, β-SG 28, γ,-SG 45, δ-SG 32, disferlina 37 e calpaína-3 9. Foi observada redução de α-SG em 7 pacientes, β-SG em 4, γ-SG em 2 e δ-SG em 8. Houve deficiência de α-SG em 7 pacientes, β-SG em 6, γ-SG 9, δ-SG em 5, disferlina em 8 e calpaína-3 em 5. Os pacientes foram classificados de acordo com a ID em deficiência de SG em 18 casos, disferlina em 8 e calpaína-3 em 5. A hipertrofia de panturrilhas foi observada apenas no grupo com deficiência de SG. O grupo com deficiência de disferlina teve maior número de mulheres acometidas e a idade de início dos sintomas foi mais tardio em relação aos grupos com deficiência de SG e calpaína-3. O grupo com deficiência de calpaína-3 ocorreu apenas em pacientes do sexo masculino, a idade do início dos sintomas foi menor e teve maior fraqueza muscular.

PALAVRAS-CHAVE: distrofias musculares de cinturas, imunoidentificação, sarcoglicano, disferlina, calpaína-3.

The cytogenetic localization of the gene of the Duchenne muscular dystrophy (DMD) in the short arm of chromosome X[1], locus Xp21[2], with posterior cloning of the DNA[3], codification[4] and identification of the gene product, dystrophin[5] and subsequent characterization of the dystrophin glycoprotein complex (DGC)[6,7] have brought great advances in the briefing of molecular pathogeneses of muscular dystrophies. The DGC is a multisubunit complex of proteins, which form a structural linkage between the cytoskeleton (F-actin) and the extracellular matrix (laminin-α2)[8]. The integral proteins

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that comprise the DGC are structurally organized into sub-complexes, formed by the dystrophin, the dystroglycan complex (α and β subunits), the sarcoglycan (SG) complex (α, β, γ, δ and ε subunits), α-dystrobrevin, syntrophins and sarcospan[9]. At least six forms of muscular dystrophy arise from primary mutations in genes encoding components of this complex[10,11]. With the identification of these genes and their product, the limb-girdle muscular dystrophies (LGMD) where classified in autosomal dominant (LGMD1) and recessive (LGMD2). Pathogenic mutations in the SG complex components determine a group of autosomal recessive limb girdle muscular dystrophies (LGMD2) known as sarcoglycanopathies: the γ, α, β and δ sarcoglycanopathy, genetically classified as LGMD2C[12], 2D[13], 2E[14] and 2F[15] respectively .The sarcoglycanopathies present a variable clinical features and is characterized by the biochemical deficiency of its subunits, independently of any primary gene defects[16].

Among the LGMD where expression of sarcoglycans is normal, other genes are involved, that can cause defects or deficiencies in sarcolemmal proteins: dysferlin (LGMD2B)[17], caveolin-3 (autosomal dominant limb girdle muscular dystrophy - LGMD1C)[18]; cytoplasmatic proteases: calpain-3 (LGMD2A)[19]; cytoplasmatic proteins associated with organelles: TRIM32 (LGMD2H)[20], fukutin related protein (FKRP) (LGMD2I)[21]; sarcomeric proteins: telethonin (LGMD2G)[22], titin (LGMD2J)[23], myotilin (LGMD1A)[24], filamin C (LGMD1F)[25]; and nuclear membrane proteins: lamin A/B (LGMD1B)[26].

Therefore, the limb-girdle muscular dystrophy (LGMD) becomes a clinically and genetically heterogeneous group of degenerative muscular diseases where the clinical, laboratory, electromyographic, histopathological and immunohistochemical have turned to be of great importance in the guideline of the specific genetic study. These made us to carry through this work, with the intention to improve the diagnosis in a heterogeneous group of patients with LGMD.

METHOD

We selected 56 patients with LGMD diagnostic and normal dystrophin by immunofluorescence (rod domain, carboxy and amino terminal) admitted to the Neuromuscular Unit, from January 1976 to May 2001. The patients were submit to clinical evaluation, serum muscle enzymes, electromyography, muscle biopsy, and the immunoidentification (ID) of α-sarcoglycan, β- sarcoglycan, γ-sarcoglycan, δ-sarcoglycan, dysferlin and calpain-3.

Clinical evaluation – We collected data regarding gender distribution, family history, age and mode of onset, muscle strength, muscle atrophy and hypertrophy, functional abilities, and progression of the disease. To assess muscle strength we used a manually muscle testing of the British Medical Research Council (MRC) scale converted to 0-7 point system as follows: 0=0, 1=1, 2=2, 3=3, 4(-)=4, 4=5, 4(+)=6, 5=7[27]. The proximal and distal muscles of the upper and lower limbs were tested. The functional grade was classified the Vignos and Archibald scale[28].

Muscle enzymes – The serum muscle enzymes activity to creatine kinase (CK) was performed in 49 cases, lactic dehydrogenase (LDH) in 25 cases, alanine aminotransferase (ALT) in 40 cases, aspartate aminotransferase (AST) in 25 cases and aldolase in 19 cases. The plasmatic levels were registered as time fold increased above the normal limit.

Electromyography – The electromyography (EMG) was performed in 50 patients and was classified as normal, myopathic, and mixed.

Muscle biopsy – Open muscle biopsies were taken from deltoid, biceps or quadriceps. All samples were frozen in liquid nitrogen and cryostat sections stained histologically and histochemically according to standard procedures[29]. The following features were assessed: variation in muscle fiber diameter, the distribution of atrophied and hypertrophied fibers; fiber degeneration and regeneration processes, architectural changes, connective and fat tissue increase and inflammatory changes.

Immunocytochemistry – Indirect immunofluorescence microscopy of 4 μ cryosections from skeletal muscle biopsy specimens was performed[30]. The samples were incubated against monoclonal antibodies to α-SG diluted 1:20 (Novocastra NCL-50DAG, Newcastle upon Tyne, UK), β-SG diluted 1:100 (Novocastra NCL-b-SARC, Newcastle upon Tyne, UK), γ-SG diluted 1:10 (Novocastra NCL-g-SARC, Newcastle upon Tyne, UK), δ-SG diluted 1:25 (Novocastra NCL-d-SARC, Newcastle upon Tyne, UK), and dysferlin diluted 1:10 (Novocastra/NCL-Hamlet, Newcastle upon Tyne, UK). The immunofluorescence to α-SG e γ-SG was realized in all muscle samples, and to β-DG in 48, to δ-SG and dysferlin in 45, and to β-SG in 38. The primary antibodies were detected with an appropriate biotinylated secondary antibody diluted 1:500 (Amershem/RPN 1025,Little Chalfont, UK), followed by streptavidin conjugated to flourescein (Amershem/RPN 1232, Little Chalfont, UK, 1:1000). The immunofluorescence intensity was classified as: 0 = absent: no labeling on any muscle fibers; + = traces: faint fluorescence on occasional fibers; majority of fibers negative; ++ = reduced: moderately and uniformly decrease fluorescence; +++ = normal: uniformly intensity fluorescence. The im-

munofluorescence to dysferlin was deficient only when the fluorescence was absent.

Western blot – The western blot was performed in patients with normal labeling to α-SG, β-SG, γ-SG, δ-SG, and dysferlin. Only thirteen muscles samples were available. The muscle proteins were extracted in treatment buffer containing 0.125 mol/l Tris-HCL buffer pH 6.4, 10% glycerol, 4% SDS, 4 mol/l urea, 10% mercaptoethanol and 0.001% bromophenol blue (final pH of the treatment buffer was 6.8). Soluble proteins were separated using a SDS-PAGE gel 10% and the transferred into nitrocellulose membrane. The visualization of blotted proteins nitrocellulose strips were blocked in 5% milk powder in a pH 8 buffer containing 10 mmol/l Tris-HCL, 0.15mol/l NaCl and 0.05% Tween 20 (TBST). Blots were probed with antibodies against to calpain-3 diluted 1:100 (Novocastra/NCL-12A2, Newcastle upon Tyne, UK) and visualized using peroxidase-conjugated anti-mouse secondary antibody diluted 1:1000 (Amersham/NA931, Little Chalfont, UK) followed by exposure to freshly prepared 0.05% diaminobenzidine and 0.1% H_2O_2[31]. Only the absence of band to calpain-3 was considered deficient.

Statistical analysis – Chi-square and Mann-Whitney tests were used to analyze the relation between the presences of abnormalities in the ID groups.

RESULTS

The α-SG was normal in 42 patients, β-SG in 28, γ-SG in 45, δ-SG in 32, dysferlin in 37 and calpain-3 in 9. There was a reduction in the α-SG in 7 patients, β-SG in 4, γ-SG in 2, and δ-SG in 8. There was deficiency of α-SG in 7 patients, β-SG in 6, γ-SG in 9, δ-SG in 5, and dysferlin in 8. The calpain-3 was absent in 5 patients (Table 1).

The patients were classified according to the type of protein deficiency. The Group A, characterized by reduction or deficiency of one or several the SG-complex, was reported in 18 patients (Fig 1); group B, characterized by dysferlin deficiency, in 8 (Fig 2); group C with calpain-3 deficiency in 5 (Figs 3 and 4). The group D, not classified, did not show any deficiency. The group E with 17 cases had a non-conclusive evaluation due to insufficient material for the tests and was not included in the statistical analysis (Table 1).

Clinical evaluation – Both male and female patients were affected, with a preponderance of male patients. There was a significant statistical relevance among the groups with dysferlin and calpain-3 deficiency (p=0.005).

Most of patients were sporadic cases. Patients with family history and, or consanguinity of the parents were more common in the dysferlin deficiency group (Table 2), but without statistical significance (p>0.05). An autosomal dominant pattern was observed only in a female patient of group D.

The mean age of onset and at evaluation was significantly higher in dysferlin deficiency group (group B) than among sarcoglycans (p= 0.014) and calpain-3 (p = 0.010) deficiency groups (Table 3). The diseases duration do not showed statistical significance among the ID groups (p>0.05).

The symptoms at presentation occurred by weakness of the muscles of the lower limbs in 29 patients, upper limbs in 6 and both in 4 (Table 3). No statistical difference occurred among the ID groups (p>0.05).

The proximal muscle atrophy was observed in all ID groups. Only the dysferlin deficiency group (group B) does not showed distal muscle atrophy (Table 4). Facial weakness occurred in all the groups of ID. In the sarcoglycanopathy group it was observed only in the cases with specific deficiency of γ-SG. The calf hypertrophy was observed in 4 patients with SG complex deficiency. The calpain-3 deficiency group (group C) presented the lowest muscle strength tests, but without statistical significance. The waddling gait and Gowers sign were commons features in all ID groups. The loss of gait occurred only in SG complex deficiency (group A) and the non-classified (group D) patients. Most of patients showed at evaluation gait impairment, difficulties in running or climbing stairs (Table 4).

Muscle enzymes – The mean elevation of CK and AST in the serum was more expressive in dysferlin deficiency patients; and of LDH, ALT and aldolase in SG complex deficiency patients, but without statistical significance (Table 5).

Electromyography – The myopathic electromyographic pattern was a common finding, observed in 31 patients, but the neuromyopathic pattern occurred in all ID groups (Table 6).

Muscle biopsy - Histopathology – Fiber size variation was encountered in 38 (97.1%) of muscles specimens. Type 1 and 2 fiber atrophy and hypertrophy were more frequent in the calpain-3 deficiency (group A). Scattered angulated fibers were seen in 24 (61.5%) of specimens, and were more common in calpain-3 deficiency group (group A).

Table 1. Immunocytochemical and western blot analysis.

Cases	α-SG	β-SG	γ-SG	δ-SG	Dysferlin	Calpain-3
Group A – 18 patients (32,1%)						
1	++	ND	+++	+++	+++	ND
2	++	ND	+++	ND	ND	ND
3	++	ND	+++	+++	+++	ND
4	++	+++	+++	+++	+++	ND
5	+	ND	0	+++	+++	ND
6	++	+++	0	++	+++	+++
7	+++	+++	+++	++	+++	ND
8	+++	+++	+++	++	+++	ND
9	0	0	0	0	+++	ND
10	+	0	+	+	+++	ND
11	+	++	+	++	+++	ND
12	0	0	0	0	+++	ND
13	++	+	+	++	+++	ND
13	+	0	0	+	+++	ND
15	+	+	+	+	+++	ND
16	+++	++	+++	++	+++	ND
17	++	++	++	++	+++	ND
18	+++	++	++	++	+++	ND
Group B – 8 patients (14,3%)						
19	+++	+++	+++	+++	0	ND
20	+++	+++	+++	+++	0	ND
21	+++	+++	+++	+++	0	ND
22	+++	+++	+++	+++	0	ND
23	+++	+++	+++	+++	0	ND
24	+++	+++	+++	+++	0	ND
25	+++	+++	+++	+++	0	ND
26	+++	+++	+++	+++	0	ND
Group C – 5 patients (8,9%)						
27	+++	+++	+++	+++	+++	0
28	+++	+++	+++	+++	+++	0
29	+++	+++	+++	+++	+++	0
30	+++	+++	+++	+++	+++	0
31	+++	+++	+++	+++	+++	0
Group D – 8 patients (14,3%)						
32	+++	+++	+++	+++	+++	+++
33	+++	+++	+++	+++	+++	+++
34	+++	ND	+++	+++	+++	+++
35	+++	+++	+++	+++	+++	+++
36	+++	+++	+++	+++	+++	+++
37	+++	+++	+++	+++	+++	+++
38	+++	ND	+++	+++	+++	+++
39	+++	ND	+++	+++	+++	+++
Group E – 17 patients (30,4%)						
40	+++	+++	+++	+++	+++	ND
41	+++	+++	+++	+++	+++	ND
42	+++	+++	+++	+++	+++	ND
43	+++	+++	+++	+++	+++	ND
44	+++	+++	+++	+++	+++	ND
45	+++	+++	+++	+++	+++	ND
46	+++	+++	+++	+++	+++	ND
47	+++	ND	+++	ND	ND	ND
48	+++	ND	+++	ND	ND	ND
49	+++	ND	+++	ND	ND	ND
50	+++	ND	+++	ND	ND	ND
51	+++	ND	+++	ND	ND	ND
52	+++	ND	+++	ND	ND	ND
53	+++	ND	+++	ND	ND	ND
54	+++	ND	+++	ND	ND	ND
55	+++	ND	+++	ND	ND	ND
56	+++	ND	+++	ND	ND	ND

0, absent; +, traces; ++ , reduction; +++ , normal; ND , not done; group A , sarcoglycanopathy; group B , dysferlinopathy; group C , calpainopathy; group D , not classified; group E , non conclusive.

Table 2. Gender and family history by immunoidentification groups.

Immunoidentification groups	A	B	C	D	Total
Number of patients	18	8	5	8	39
Female	8	7	–	3	18
Male	10	1	5	5	21
Family history	7	5	2	4	18

A, sarcoglycanopathy; B, dysferlinopathy; C, calpainopathy; D, not classified.

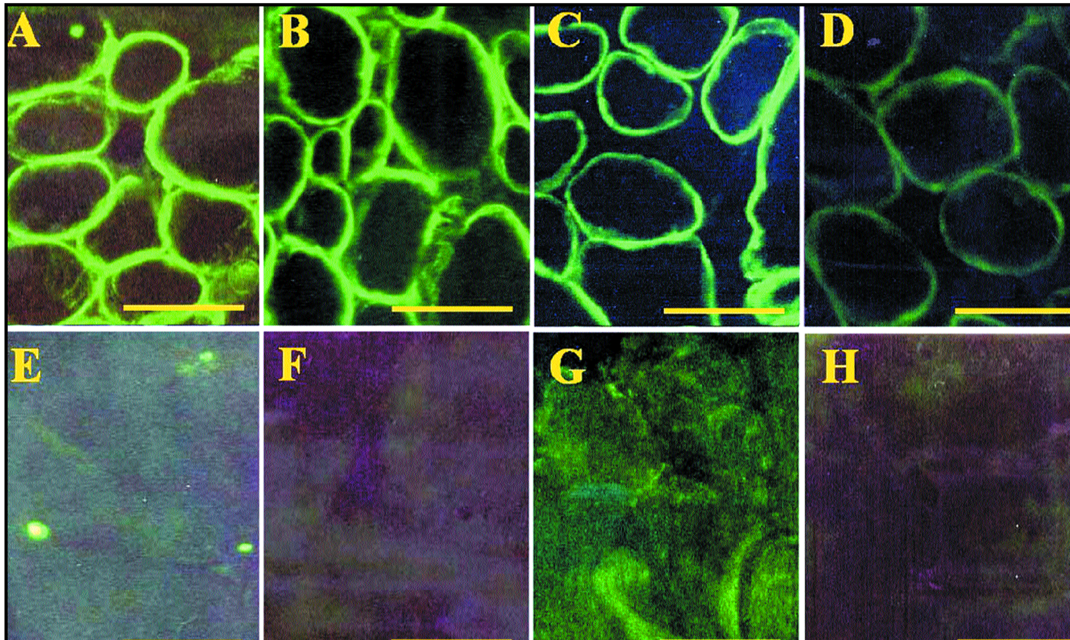

Fig 1. Sarcoglycan deficiency (Case 12, immunofluorescence). A, Dystrophin carboxyl terminal; B, Dystrophin amino terminal; C, Dystrophin Rod domain; D, Dyspherlin; E, α-Sarcoglycan; F, β-Sarcoglycan; G, γ-Sarcoglycan; H, δ-Sarcoglycan. (Bar 100μ in A,B,C,D; 25μ in E,F,G,H).

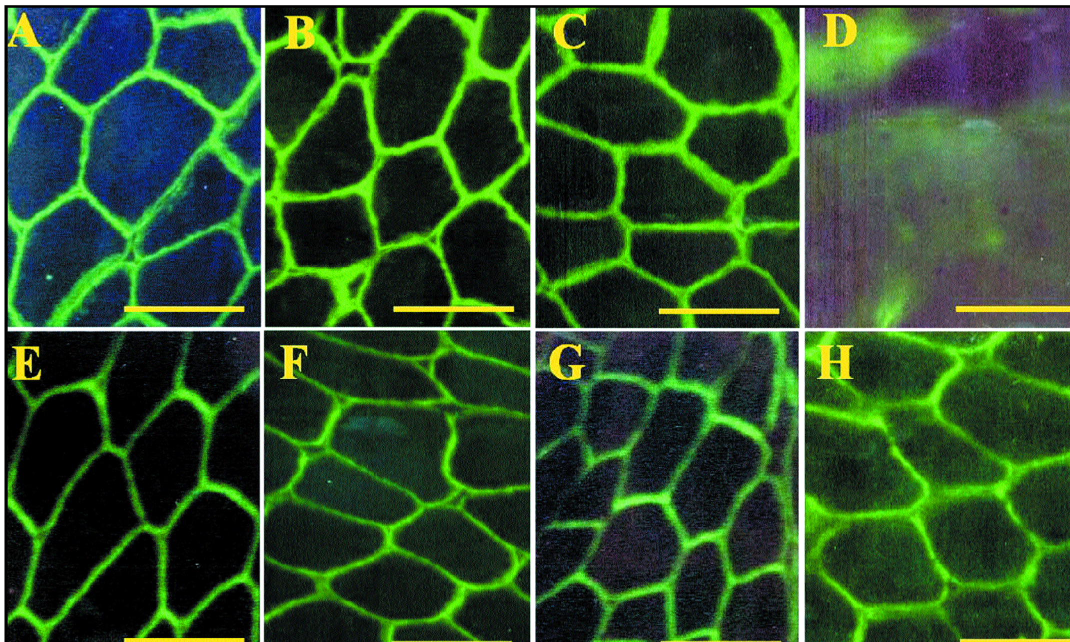

Fig 2. Dyspherlin deficiency (Case 22, immunofluorescence). A, Dystrophin carboxyl terminal; B, Dystrophin amino terminal; C, Dystrophin Rod domain; D, Dyspherlin; E, α-Sarcoglycan; F, β-Sarcoglycan; G, γ-Sarcoglycan; H, δ-Sarcoglycan. (Bar 100μ in A,B,C,E,F,G,H; 25μ in D).

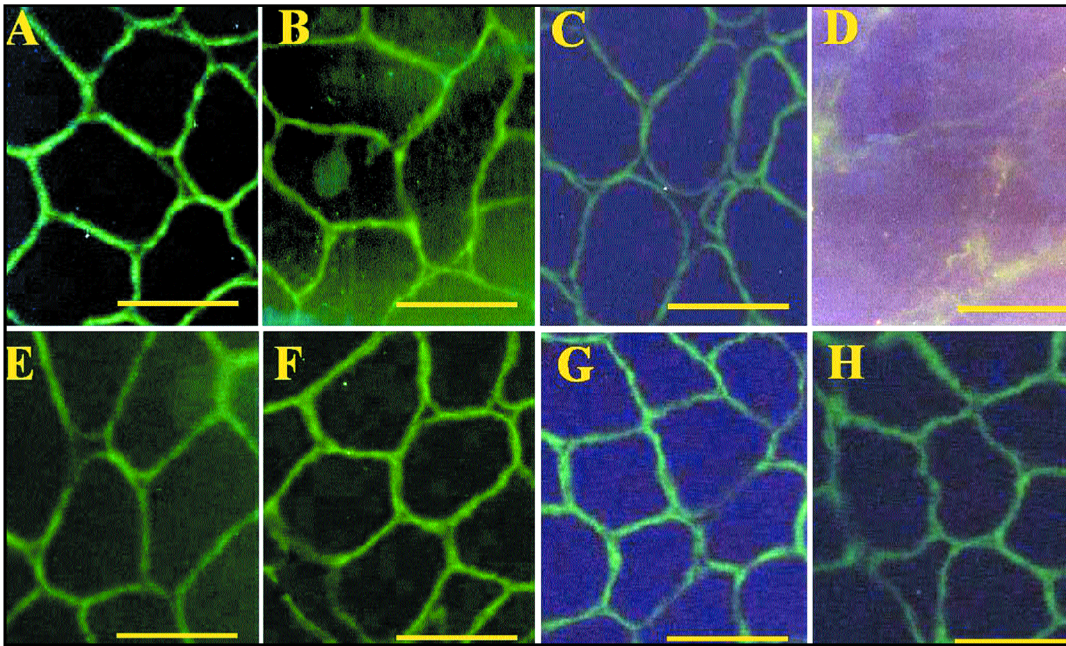

Fig 3. Calpain-3 deficiency (Case 28, immunofluorescence). A, Dystrophin carboxyl terminal; B, Dystrophin amino terminal; C, Dystrophin Rod domain; D, Dyspherlin; E, α-Sarcoglycan; F, β-Sarcoglycan; G, γ-Sarcoglycan; H, δ-Sarcoglycan. (Bar 100μ in A,B,C,E,F,G,H; 25μ in D).

Fig 4. Calpain-3 deficiency (Case 28, Western blot). A, Normal control; B, Absent band (Patient).

Small group atrophy was found only in 5 specimens. Type 1 and 2 fibers predominance and vacuoles were uncommon findings. Central nuclei and nuclear clumps were found more frequently in the groups with dysferlin deficiency and also in the non-classified group. Fibers with necrosis were found more frequently in the calpain-3 deficiency group. Basophilic and segmented fibers were more frequently reported in the group with dysferlin deficiency and in the non-classified group. The perivascular inflammatory infiltrates was more common in the group with SG complex deficiency. The increase of fat and fibrous connective tissues was more frequent in dysferlin deficiency patients (group B). The ring fibers, lobulated fibers, and whorled fibers were more common in the group with calpain-3 deficiency. No statistically relevance were found among the ID groups and the several abnormal specific histological findings ($p>0.05$) (Table 7).

Regarding the histological diagnosis, the myopathic pattern was the most common finding in all ID groups. In some of the samples, a suggestion of a neurogenic processes was seen in patients with SG complex (group A), dysferlin (group B) and calpain-3 deficiency (group C). Also, the mixed pattern (myopathy with denervation findings) occurred in the groups with complex SG deficiency and non-classified. No statistical significance was found among the ID groups ($p>0.05$).

Table 3. Mean age of onset of the symptoms and at evaluation, symptom at presentation by immunoidentification group.

Immunoidentification groups	A	B	C	D	Total
Age of onset	12.36 (1.2 – 36)	27.12 (10 – 57)	7.34 (0.7 – 17)	17.95 (1 – 39)	15.89 (0.7 – 57)
Age at evaluation	19.28 (2 – 43)	33.87 (17 – 61)	17.40 (8 – 24)	27.40 (12 – 42)	23.72 (2 – 61)
Diseases duration	6.35 (0.7 – 28)	6.81 (1.5 – 23)	10.58 (5 – 23)	9.80 (0.4 – 25.5)	7.68 (0.4 – 28)
Symptom at presentation					
Weakness					
Upper limbs	3	2	1	–	6
Lower limbs	13	6	3	7	29
Both limbs	2	–	1	1	4

A, sarcoglycanopathy; B, dysferlinopathy; C, calpainopathy; D, not classified.

Table 4. Clinical findings in the neurological examination according the immunoidentification groups.

Immunoidentification groups	A	B	C	D	Total
Muscular atrophy					
Upper limbs					
Proximal	10	5	5	7	27
Distal	4	–	2	3	9
Lower limbs					
Proximal	11	3	4	6	24
Distal	3	–	2	3	8
Calf Hypertrophy	4	–	–	–	4
Muscle force graduation (mean)					
Upper limbs					
Proximal	4.94 (2 – 7)	5.13 (3 – 7)	4.00 (3 – 5)	4.88 (3 – 6)	4.85 (2 – 7)
Distal	6.61 (4 – 7)	6.75 (5 – 7)	5.80 (4 – 7)	6.13 (5 – 7)	6.44 (4 – 7)
Lower limbs					
Proximal	4.83 (2 – 7)	4.63 (3 – 7)	4.20 (3 – 6)	4.50 (4 – 5)	4.64 (2 – 7)
Distal	6.78 (5 – 7)	6.63 (5 – 7)	5.00 (4 – 7)	6.25 (4 – 7)	6.41 (4 – 7)
Facial weakness	2	1	1	2	6
Gait type					
Normal	2	2	1	1	
Waddling	15	6	4	6	31
Unable to walk	1	–	–	1	2
Gowers sign					
Present	13	6	5	7	31
Unable to perform	2	–	–	1	3
Vignos functional scale					
1 – 4	17	8	5	7	37
7	–	–	–	1	1
9	1	–	–	–	1

A, sarcoglycanopathy; B, dysferlinopathy; C, calpainopathy; D, not classified.

Table 5. Mean muscular enzymes by immunoidentification group.

Immunoidentification group	A	B	C	D	Total
Muscular enzymes					
CK	17.39 (0 – 66)	23.85 (0.8 – 46)	10.45 (0.4 – 20.5)	12.26 (2.6 – 23)	17.41 (0 – 66)
LDH	1.86 (0 – 11)	1.38 (0 – 3.3)	0.10 (0 – 0.1)	0.52 (0 – 0.2)	1.39 (0 – 11)
AST	1.13 (0 – 4)	16.01 (0 – 80)	0.70 (0 – 1.4)	4.08 (0 – 20)	5.45 (0 – 1)
ALT	0.68 (0 – 2)	0.37 (0 – 1.3)	1.00 (0 – 2)	0.16 (0 – 0.5)	0.55 (0 – 2)
Aldolase	3.21 (0 – 12)	–	0.10 (0 – 0,2)	–	2.49 (0 – 12)

A, sarcoglycanopathy; B, dysferlinopathy; C, calpainopathy; D, not classified; CK, Creatine kinase; LDH, Lactic dehydrogenase; AST, Aspartate aminotransferase; ALT, Alanine aminotransferase.

Table 6. Electromyographic pattern by immunoidentificatoin group.

Immunoidentification group	A	B	C	D	Total
Electromyography pattern					
Myopathic	15	7	2	7	31
Mixed	1	1	1	1	4

A, sarcoglycanopathy; B, dysferlinopathy; C, calpainopathy; D, not classified.

Table 7. Immunoidentification groups and histopathology.

Immunoidentification group	A	B	C	D	Total
Fiber diameter variation	18	8	5	7	38
Type 1 fiber atrophy	15	7	5	7	34
Type 2 fiber atrophy	14	6	4	5	29
Type 1 fiber hypertrophy	12	6	4	5	27
Type 2 fiber hypertrophy	12	6	4	7	29
Atrophic angulated fibers	10	5	5	4	24
Small group atrophy	1	1	–	3	5
Type 1 fiber predominance	3	–	2	1	6
Type 2 fiber predominance	1	–	–	1	2
Central nuclei	12	7	3	7	29
Nuclear clumps	2	3	1	3	12
Necrotic fibers	8	6	4	5	23
Basophilic fibers	4	3	1	4	12
Segmentation fibers	7	2	3	4	16
Perivascular cellular infiltrates	8	2	1	2	13
Increase fat connective tissue	8	6	3	4	21
Increase connective tissue	11	6	3	5	24
Ringed fibers	2	1	2	1	6
Lobulated fibers	7	4	3	1	15
Whorled fibers	5	3	4	2	14
Vacuoles	–	1	2	1	4
Rimmed vacuoles	1	1	–	–	2

A, sarcoglycanopathy; B, dysferlinopathy; C, calpainopathy; D, non-classified.

DISCUSSION

The identification of the forms of LGMD with autosomal recessive inheritance is often difficult to be established, considering the great number of sporadic cases, the lack of convincing data in family history and its great clinical similarity with the Duchenne and Becker muscular dystrophies[32]. These cases had no correlation among the groups of ID and family history, but it was more frequent in the group of the dysferlinopathy[33]. The lack of changes in the ID of the cases of the autosomal dominant trait corroborates the literature data[34]. The great variability in the age of onset difficult the characterization of a typical pattern to each type of LGMD, and the mean age of onset can be common to more than one. We have verified that the cases with dysferlin deficiency showed a later onset[33,35] and the degrees of deficiency of the sarcoglycan protein complex proteins did not interfere in a meaningful way in the age at onset either[36].

The classic presentation of the symptoms has been the weakness of the hip-girdle muscles, but can also present as involvement of the shoulder girdle or lower-limb distal muscles, muscular pain, and exercise intolerance[20,36,37]. Some specific patterns can be observed in the calpainopathy (LGMD2A), by involvement of posterior limb-girdle and trunk muscles; in the dysferlinopathy (LGMD2B), by involvement of the posterior compartment of the legs[33,38,39]; in the telethoninopathy (LGMD2G) and titinopathy (LGMD2J), by involvement of the anterior compartment of leg[22,34]. The facial weakness can be observed in advanced stages of the illness in the sarcoglycanopathies (LGMD2C-2F)[40], and occasionally in the calpainopathy (LGMD2A) and telethoninopathy (LGMD2G)[22,37]. The calf hypertrophy is a common finding among the sarcoglycanopathies but can occasionally be observed in the early stages of the calpainopathy and dysferlinopathy[35]. In our cases the muscular involvement was unspecific, the facial musculature took place in intermediate stages of the disease in all groups of ID, and the calf hypertrophy was observed in the group of SG-complex deficiency.

The muscle enzymes are usually more elevated in the pre-clinical or initial stages of the illness and tend to present gradual decline according to the evolution of the disease, because of the gradual loss of muscular bulk[41]. The increase is more discrete in the autosomal dominant forms (LGMD1A - E) and the telethoninopathy (LGMD2G), but it is generally important in others recessive forms[22,34,42]. Important variations can occur independent of the stage of the disease[43]. The mean serum levels of muscular enzymes were similar for all groups, not being possible to differentiate them.

The progressive loss of the muscle fibers results in the generation of myopathic motor unit potentials in the electromyography. However, neurogenic motor unit potentials can also be observed in areas with clustering or fiber hypertrophy, owing to motor unit remodeling caused by segmentary necrosis process, which can isolate the distal portions of the muscular fibers from the myoneural endplate[43]. The presence of neurogenic motor unit potentials has described in calpainopathy, dysferlinopathy and sarcoglycanopathies[39,44,45]. The dystrophic changes at muscle biopsy, characterized by variation in muscle fiber size, necrotic/regenerating process, and increase of the endomysial and perimysial connective tissue, is the landmark of LGMD[46].

The variation in muscle fiber size is generally light to moderate degree in the calpainopathy and dysferlinopathy, and more intense in the sarcoglycanopathies[33,45], in the telethoninopathy (LGMD2G)[47] and LGMD2H[48]. Type 1 fiber predominance can be more intense in the sarcoglycanopathies (LGMD2C-2F)[47] and calpainopathy (LGMD2A)[42,49]. The degenerating and regenerating process do not characterize any specific form in LGMD. The proliferation of the conjunctive and fat connective proliferation usually tends to be more intense in the final stages of the disease[46]. The cellular reactions in the muscular dystrophies are unspecific, and generally vary according to the degree of muscular necrosis, because of the activation and release of the complement[46]. Some perivascular inflammatory reaction can be very similar to inflammatory myopathy, as observed in the sarcoglycanopathies[47] and dysferlinopathy[50]. The structural alterations in the majority of the autosomal recessive forms of the LGMD are of little intensity and unspecific, with exception of the telethoninopathy (LGMD2G) and the titinopathy (LGMD2I), where there have been reported the formation of rimmed vacuoles[23,47]. Notwithstanding, the existence of rimmed vacuoles is a non-specific finding and has been reported in many neuromuscular diseases, like the inclusion body myositis, spinal muscular atrophies and peripheral neuropathies[51]. The segmentary necrosis processes, can isolate the distal portions of the muscular fibers from the myoneural endplate, and remodeled the motor unit[43].

Therefore, angulated atrophic fibers, small groups of atrophic fibers, and nuclear clumps can occasionally be observed in the muscular dystrophies, especially in facioescapulohumeral dystrophy and the LGMD syndromes[46].

In the sarcoglycanopathies (LGMD2C-2F), the modifications of the SG-complex may also cause a secondary dystrophin deficiency, making the separation from the dystrophinopathies very difficult. Therefore, we chose to include only the cases with normal dystrophin[52,53]. The ID of α-SG has been used as the main mark the sarcoglycanopathies, because of the structural alterations of complex SG caused for the mutations in the genes of these proteins[52]. The α-sarcoglycan deficiency varies of 9% to 30% of the cases, depending on the studied population[16,53,54]. Considering the possibility of the preservation of this marker in the form LGMD2C, we used the remaining ID markers to the proteins of the SG complex. This choice must have contributed to the identification of a larger number of cases when compared to the literature data[16,54]. The alterations of complex SG must be analyzed with caution; therefore they are normally not followed by mutations of the genes of the proteins of complex SG[16]. This fact make possible the occurrence of a secondary deficiency of the complex by other types of mutations, as observed in LGMD2I, where it occur secondary deficiency of α-distroglycan and merosin[55]. In β and γ-sarcoglycanopathies (LGMD2E and 2F), the deficiency of proteins of complex SG occurs in a much more uniform way, and does not present a specific pattern[16,56,57]. In the α-sarcoglycanopathy (LGMD2D), also a larger correlation with the degree of deficiency of the α-sarcoglycan can exist[16,58]. Only in the γ-sarcoglycanopathy (LGMD2C) the deficiency isolated of protein γ-SG seems to be a specific result, which suggests a strong correlation of this disease[59]. However, there is a better degree of correlation with mutations of complex SG concerning the cases with important deficiency exist[16,52].

The immunocytochemical analysis of dysferlin proved to be a difficult technique to interpret, due to the weak fluorescence intensity. The dysferlinopathy corresponds to 5 to 55% of the LGMD with preservation of the complex SG[32,33,35,53,60]. The calpain-3, for being a cytoplasmatic enzyme, has been detected only through the western blot technique. The calpain-3 deficiency has been frequently correlated with the protein gene mutations. However, secondary reductions had been also observed in the cases with mutations in the gene of the dysferlin (LGMD2B) and titin (LGMD2J)[23]. Other alterations also can be caused due to the muscle biopsy storage time, the amount of present protein and by the process of homogenization of muscular tissue. Distortions of the lane of blot difficulties the characterization of the calpain-3 band reduction, probably caused by contamination with the mountant medium. The mutations in the calpain-3 gene have been observed between 9 to 40% LGMD cases[35,53,60,61]. However, is estimated that 10% of the mutations of the gene of calpain-3 are not detected by the molecular techniques currently used[62].

The immunocytochemical and western blot analysis were useful methods to classify the LGMD patients. The sarcoglycan deficiency was more frequent, followed of the dysferlinopathy and calpainopathy. Heaven the clinical and laboratory findings were very similar between the ID groups, the dysferlin deficiency patients had more delayed onset, and occurred more in female, and the calpain-3 deficiency patients occurred only in males and had greater impairment in the muscle strength.

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