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RESEARCH ARTICLE

Limb-Girdle muscular dystrophy in Brazilian children: clinical, histological and molecular characterization

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ABSTRACT

Background: Limb-girdle muscular dystrophies (LGMD) are a heterogeneous group of genetic muscular dystrophies, involving autosomal recessive (LGMD R) and dominant (LGMD D) subtypes. The recessive forms are far more common than dominant, particularly in children. The clinical course in this group is characterized by progressive proximal weakness, initially in pelvic and after in shoulder-girdle musculature, varying from very mild to severe degree. Significant overlap of clinical phenotypes, with clinical and genetic heterogeneity, constitutes the rule for this group of diseases. Muscle biopsies are useful for histopathologic and immunolabeling studies, and DNA analysis is the gold standard to establish the specific form LGMD.

Objectives: The aim of this study was to characterize the clinical, histological and molecular aspects in children with LGMD who attend a big public neuromuscular centre in our country to determine the frequency of different forms.

Results: Thirty-nine patients were enrolled in this study and 34 were classified as LGMD. The female to male ratio was 3:1. The mean age at onset of the disease was 6 years (2-13 years). The first sign of the disease were related to a proximal muscular involvement in lower limb, including: frequent falls (22 patients), difficulty in climbing stairs (12 patients) and difficulty to rise from the floor (2 patients). In three patients, the first sign was walking on tiptoe. All patients showed normal milestones and were able to walk before 18 months of age. CK level was elevated in all cases. Of the 34 cases defined LGMD, the frequency found were: 15(44%) Sarcoglycanopathies (LGMD Sarcoglycan-related); Five (15%), Calpainopathy (LGMD Calpain3-related); Five (15%) Dysferlinopathy (LGMD Dysferlin-related) and two (6%) LGMD FKRP-related. In 7/34 patients (21%) it was not possible to define the specific subtype, despite the techniques used. Calf hypertrophy, winging of the scapula and scoliosis were the most characteristic signs in Sarcoglycanopathies. In FKRP-related forms, calf hypertrophy was also observed. Atrophy of posterior compartment of thighs is frequent in children with Dysferlinopathy and could suggest the diagnosis. In Calpainopathy winging of the scapulae and contractures in Achilles tendons were the more important findings. Muscle biopsy showed a dystrophic pattern in all cases, more intense in Sarcoglycanopathies and LGMD FKRP-related.

Conclusions: Our study on LGMD in children demonstrates that Sarcoglycanopathy is the most frequent form of LGMD, particularly in most severe forms. Because of clinical findings overlapping and unspecific pattern in muscle biopsy, for a correctly identify of the subtype, mutation screening through a myopathies panel or exome sequencing is essential.

Descriptors: 1.Muscular dystrophy; 2. Muscular dystrophy Limb-Girdle/classification; 3. Muscular dystrophy Limb-Girdle/genetic; 4.Muscular dystrophy Limb-Girdle/pathology; 5. Child; 6. Biopsy.

Keywords: LGMD/ children/ Brazilian/ muscle biopsy.

Introduction

Muscular Dystrophies (MD) are a heterogeneous group of progressive, genetically determined myopathies, in which there is a primary involvement of the skeletal muscles leading to muscular weakness, hypotonia, reduction or abolition of osteotendinous reflexes, as well as muscular atrophy, fibrotendinous retractions and skeletal deformities. The respiratory muscles are affected to a variable degree and, in some subtypes, there is also cardiac involvement. Muscular dystrophies (MD) show wide clinical heterogeneity, ranging from very severe, rapidly progressive forms that begin at birth, to mild forms that begin in adolescents or adults with a slowly progressive course[1].

Traditionally, muscle biopsy and histopathology along with special pathology techniques such as immunohistochemistry or immunoblotting were used for the diagnosis of muscular dystrophies. In all of them, shows the same pathological basis characterized by variability in fiber size, proliferation of endomysial and perimysial connective tissue, increase in internalized nuclei, presence of necrosis to a variable degree, macrophage and aspects of degeneration with some regeneration[2].

Most muscular dystrophies result from changes in the dystrophin-associated glycoprotein (CDG) complex proteins, which interconnect the muscle fiber membrane with the extracellular matrix, in order to provide stability of the muscle fiber so that it does not rupture during repeated cycles. of muscle contraction and relaxation[3,4].

Limb-girdle muscular dystrophies (LGMD) represents a heterogeneous group of progressive muscular dystrophies resulting from different molecular defects that cause protein deficiency in muscle fibers and can manifest at any age, most commonly in adolescents and young adults, and can also occur in childhood from the second year of life, when the development of independent walking has already occurred (muscular dystrophies that begin at birth or in the first year of life belong to the category of Congenital Muscular Dystrophies (CMD) and are accompanied by development motor delay[5].

In LGMDs, inheritance can be autosomal recessive or dominant and are clinically characterized by progressive muscular weakness of proximal predominance, generally starting in the pelvic girdle and, later, in the shoulder girdle, frequently associated with other clinical signs such as calf hypertrophy, joint retractions and skeletal deformities such as winging of scapula and scoliosis. The distal muscles may be affected, but usually only late in the progression of the disease. Involvement of respiratory muscles and cardiac muscles is described in many forms of the disease. Different degrees of increase in the serum level of creatine kinase (CK) are observed and, sporadically, there is involvement of the central nervous system (CNS) and gastrointestinal tract[6-9].

In the last 30 years, with the progress of molecular genetic, numerous genetic defects and their respective protein deficiencies in different locations in the muscle fiber have been demonstrated. The genes involved in LGMDs encode sarcolemma, sarcomere and nuclear envelope proteins, as well as, a variety of enzymes such as calpain-3 and the glycosyltransferases, which are involved in the glycosylation process of alpha-dystroglycan. Mutations in their respective genes define the

pathogenesis of different forms of the disease[10,11].

Since their initial description, LGMD have been grouped as genetic muscular diseases, with a broad clinical spectrum and varied etiology, with the aim of more conveniently differentiating this group of patients with a clinical aspects in which a precise diagnosis can't be made, of forms of Duchenne and Becker Muscular Dystrophies (DMD/BMD) and Facioscapulohumeral muscular dystrophy (FSHD). Following the proposal of a new nomenclature in 1995, LGMD that had the locus and the deficient protein identified became part of the classification, that was adopted by the World Muscle Society and the European Neuromuscular Center (GENE TABLE): the dominant forms of LGMD were called LGMD1 and the recessive forms LGMD2. Each new identification received a letter of the alphabet following the chronological order of identification, starting from the letter A. For example, the alpha, beta, gamma and delta sarcoglycanopathies, all of autosomal recessive inheritance, received the denomination LGMD2 and letters D, E, C and F, respectively. LGMD2A corresponds to Calpainopathy also called LGMD caused by calpain-deficiency, linked to 15q locus and LGMD2B is related a Dysferlinopathy or LGMD caused by dyferlin-deficiency, located at 2p13.3[12].

In 2017, during 2nd ENMC workshop on the classification and nomenclature of the LGMDs, a consensus was reached on the most useful LGMD classification system that also allowed space for further discoveries of new sub-types. The proposed subtype classification therefore follows the formula: "LGMD, inheritance (R or D), order of discovery (number), affected protein". For example, LGMD2A would be: "LGMD R1 Calpain3-related" [13]. This classification is periodically reviewed by the World Muscle Society and the updated version is made available electronically at http://www.musclegenetable.fr and http://194.167.35.195/.

Autosomal recessive forms are far more common than autosomal dominant, particularly in children. Significant overlap of clinical phenotypes, with genetic and clinical heterogeneity, constitutes the rule for this group of diseases. Muscle biopsy with histological, immunohistochemistry and western blotting studies can help to diagnosis of LGMD sub-type but, nowadays, the gold standard diagnostic method is the genetic analysis by exome sequencing for neuromuscular disease[9,10].

There are not many studies about prevalence of different forms of limb-girdle dystrophy in children. Frequency studies generally encompass childhood and adult-onset forms. In a American study on childhood-onset limb-girdle dystrophies, the following frequencies were reported among the different forms of LGMD: 16% of LGMD2A; 8% LGMD2B; 26% LGMD2C to F; 4% LGMD2I; 3% LGMD2L; 1% LGMD1A; 4% of LGMD1B, and 37% without specific subtype identification[14]. In Brazil, population frequency studies are scarce, and there isn't study including only children[15].

With the objective to investigate and to determine the frequency of different subtypes of LGMD in Brazilian children from a population from a large public teaching hospital this study was realized. We described the clinical, histological and, in some cases,

molecular aspects of the different forms of LGMD in our country.

Material and Methods

CHARACTERIZATION OF THE STUDY

This study was descriptive, characterized as a series of cases, and was developed at the Neuromuscular Diseases Outpatient Clinic of the Neurological Clinic Division of the Hospital das Clínicas of FMUSP and at the LIM 15 and 45 Laboratories of the Faculty of Medicine of the University of São Paulo- FMUSP from 2012 to 2016. This study was previously approved by the HC-FMUSP Ethics Committee.

PATIENTS

In this study, we included patients of both sexes and ages ranging from the second year of life to 18 years of age (age range relevant to care at the Child Neurology Service), and who are undergoing regular clinical follow-up at the Neuromuscular Diseases Outpatient Clinic of the Division of Clinical Neurology at the Hospital das Clínicas of FMUSP with the diagnosis of limb-girdle muscular dystrophy, which was established according to the phenotypic characteristics and the findings of the muscle biopsy. All patients had an informed consent form signed by their legal guardians.

INCLUSION CRITERIA

• Onset of symptoms noted after a period of normal motor development, including the acquisition of independent walking (up to 18 months of age), and up to 17 years, 11 months and 29 days of age.

• Muscle biopsy showing a non-specific dystrophic pattern and normal immunohistochemical analysis for dystrophin protein.

EXCLUSION CRITERIA

• Symptoms noted at birth, in the first year of life, or prior to the acquisition of walking;

• Weakness exclusively compromising the distal portions of the limbs;

• Non-progressive clinical evolution;

• Normal muscle biopsy or with specific structural changes (central-core type, nemaline bodies, nuclear centralization, mitochondrial changes, vacuoles with abnormal deposit material, etc.).

Methods

CLINICAL DATA

The following parameters were analyzed: age, sex, pedigree, age at onset of symptoms and evolution, distribution of muscle weakness and atrophy, the presence or absence of impairment of the craniofacial muscles, changes in muscle tone and deep reflexes, and the presence of changes in other neurological systems such as sensitivity, coordination and cognition were verified. The presence of skeletal changes such as muscle retractions, spinal deformity and craniofacial dysmorphic was assessed. The first author examined all children.

SUBSIDIARY EXAMS

The following auxiliary exams realized during clinical follow-up were included in the evaluation of patients: Electrocardiogram (ECG), Echocardiogram (ECHO), Holter, pulmonary function test (PFP), electroneuromyography (ENMG) and serum CK level.

Muscle biopsy

HISTOLOGICAL REACTIONS

Patients underwent muscle biopsy only once, at the time of diagnosis. In all patients, muscle

biopsy was performed on the biceps brachii muscle, under general and/or local anesthesia. In families where there was more than one affected individual, the biopsy was performed on only one affected person. Immediately after removal, the muscle fragments were mounted in cork using tissue tek, and quickly frozen in liquid nitrogen. The muscle fragments were subjected to sequential coronal sections in a cryostat at a temperature of -25oC with a thickness of 6 to 8 microns. The slides were stored at -80oC for later use. The slides with the muscle fragments were subjected to histological staining with hematoxylin & eosin (HE) and modified Gomori trichrome (GO), periodic acid-Schiff (PAS) and "oil red" O (ORO) in order to evaluate the global appearance, muscular morphology, presence of connective-fatty infiltrate, muscular necrosis and presence of deposits of PAS-positive material and fat. The histochemical reactions NADH-tetrazolium reductase (NADH), succinate dehydrogenase (SDH) and adenosine triphosphatase (ATPase) at different pHs (9.4 and 4.3) were performed to evaluate the general aspect of the internal architecture of the fibers, the oxidative activity and the distribution of different types of fibers. Muscle biopsies from all patients were evaluated to record the following aspects: variability in fiber size, proliferation of endomysial and perimysial connective tissue, deposition of adipose tissue, aspects of degeneration (necrosis) and regeneration and presence of inflammatory reaction. Such aspects were subjectively quantified as mild (+), moderate (++) and intense (+++) by the author of the work together with Dr. Edmar Zanoteli.

IMMUNOHISTOCHEMICAL REACTIONS (IH)

Immunohistochemistry reaction using antibodies to dystrophin, dysferlin, sarcoglycans, collagen-6, emerin, merosin and caveolin-3 is routinely performed in our service for the diagnosis of muscular dystrophies, and was performed in all cases included in this study. For the peroxidase immunohistochemistry technique, the unfixed muscle fragments were incubated with blocking solution (0.1% BSA, 10% normal serum, 0.5% Tween in PBS) for 30' at room temperature. After washing, the fragments were then incubated with primary antibody diluted in blocking buffer for 1-3hs at room temperature. After washing in wash buffer (0.1% BSA, 0.5% Tween in PBS), the fragments were incubated with secondary antibody for 30 min. at room temperature. The slides were then kept in 0.1% H2O2 for 30 min. to remove endogenous peroxidase and then washed in wash buffer. The ABC method was used in this way, and after washing the slides, DAB was applied for 2 to 5 min. The reaction is blocked by washing in water. The slides were then counterstained with hematoxylin, dehydrated in ascending alcohol, washed in xylene, and mounted the slides. The slides were examined under an optical microscope. As an internal control, muscle fragments were incubated with secondary antibody only.

Alternatively, for some antibodies, the indirect immunofluorescence technique was used, in which unfixed muscle fragments are maintained for 10 min. in room air and then blocked with blocking buffer (2% BSA in PBS, normal serum and 0.2% triton-X 100) for 30 min. at room temperature. Then, the fragments were incubated with primary

antibody diluted in blocking buffer for 1-3hs at room temperature. The slides were washed with PBS three times for 10 min. each, and blocked again with blocking buffer for 20 min. Then, the fragments were incubated with secondary antibody for 1 (one) hour at room temperature, and subsequently washed with PBS (4 times, 10 min each), and mounted with Vectashild (Vector Laboratories, Burlingame, CA). The slides were examined under a fluorescence microscope (Olympus DP72). As an internal control, sections were incubated with secondary antibody only. The analysis of reactions followed the following classification: normal expression of the protein, and partial reduction or absence of expression.

GENETIC ANALYSIS

In the patients in whom histological procedures did not allow defining the specific form of limb-girdle dystrophy, underwent testing for mutations in the FKRP gene. The FKRP gene has four exons; however, only exon 4 is coding. The oligonucleotide pairs used to amplify the entire exon 4 are specified in Table 1. For PCR responses, programs were used with an initial cycle of denaturation (95°C for 4 minutes), followed by 35 cycles at 95oC for 30 sec., specific annealing temperature for each primer for 30 sec., and 72oC for 2 min., with a final extension step at 72oC for 7 minutes. The PCR products were subjected to electrophoresis in agarose gel (2%) containing ethidium bromide for visualization under ultraviolet (UV) light. DNA fragments were purified using Exosap enzyme (Applied Biosystem).

Table 1: Primer sequence and screening conditions used in the FKRP coding region

Name	oligonucleotides (5′ –3′)	Gene *FKRP* fragm	PCR product	Ann temp.	PCR obs.	Met
FKRP-1F FKRP-1R	TGGTTCTGACAATCAGCTGCT CCGCGTTGTCAAATGCCTCGAA	1	274	62	-	dHPLC temp.: 65C
FKRP-2F FKRP-2R	CCCGTGTCACCGTCCTGGTGC CCCGTGTCACCGTCCTGGTGC	2	332	65	1% DMSO	dHPLC temp.: 66C
FKRP-3F FKRP-3R	GCGTCCCTCGCGCTCAGCCTTCCA GCGTCCCTCGCGCTCAGCCTTCCA	3	369	66	5% DMSO	MDE gel

Name	oligonucleotides (5' –3')	Gene *FKRP* fragm	PCR product	Ann temp.	PCR obs.	Met
FKRP-4F FKRP-4R	GCAGCTGCTGGACTTGACC TTCGC CCCAGCAGTGAGCCGCCCT CGAG	4	348	61	5% DMSO	MDE gel
FKRP-5F FKRP-5R	TGGAGGCTGCGGGCGTGC GCTACTG CCACAGGTCCACGTGCAAG TGGT	5	257	67	-	dHPLC temp.: 65C
FKRP-6F FKRP-6R	TTCCGCGTGCAGTACAGCG AAAGC CCCGAAAAACAAAGGCGA GGTT	6	313	62	-	dHPLC temp.: 64C
FKRP-7F FKRP-7R	TTCCGCGTGCAGTACAGCG AAAGC AGAGCTTCTCCACATCCAG ACA	7	339	62	-	dHPLC temp.: 64C

Legend: Fragm: fragment; ann temp., annealing temperature; temp., temperature for dHPLC

Those patients in whom the molecular study to search for mutations in the FKRP gene did not identify a mutation and who had a clinical picture compatible with the form of autosomal dominant limb dystrophy 1B (LGMD1B) were also subjected to a molecular study for the LMNA gene to search for mutations. The 12 exons of the LMNA gene were amplified in an Eppendorf thermocycler, using pairs of oligonucleotides and a previously described protocol. The sequences of the oligonucleotides used in the study are described in Table 2. In general, a final volume of 25 µl with about 100 ng of DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 200 uM dNTP, 1.5 to 2.0 mM MgCl2 , 10 pmol of each primer and 1 U Taq polymerase, was subjected to a PCR protocol adjusted for each oligonucleotide pair used. The amplified products were analyzed on a 1.5 to 2% agarose gel, stained with ethidium bromide, and visualized with ultraviolet light. The amplified products were then purified using a PCR purification kit.

Table 2: Sequence of oligonucleotides used in the study of the LMNA gene

Exon	*Forward primer /reverse primer*	Nome
1	CCCAGATCCCGAGGTCCGAC /CCTCTCCACTCCCCGCCA	1f/r
2	CAGACTCCTTCTCTTAAATCTAC /CCTAGGTAGAAGAGTGAGTGTAC	2f/r
3	CCTTCAAGTTCTTGTGTTCTGTGAC /CCTAGCCCAGCCCAAGTCTGTC	3f/r
4	GGCCTCCCAGGAACTAATTCTG /CTCCCTGCCACCATCTGC	4f/r
5	GCTGTAGCAGTGATGCCCAAC /CCAAAGCCCTGAGAAGTGAAG	5f/r
6	GCCAGGACTATGTTTAGAGCTTG /GGTCTAGTCAAGGCCAGTTG	6f/r
7	CCCCACTTGGTCTCCCTCTCC /CCCTGATGCAGCTGTATCCCC	7f/r
8	GAGGCCTCAATTGCAGGCAGGC /GAAAAGGACACTTACCCCAGC	8f/r
9	GGAGCGCTGGGGTAAGTGTC /CTCGTCCAGCAAGCAGCCAG	9f/r
10	GTAAGCAGCAGGCCGGACAAAG /CACAGGAATATTCCATGGCATC	10f/r
11	GGAGCCTGCAGGAGCCTGGAGC /GCTGCGGAAGAGAAGGCAGGCTC	11f/r
12	CTTGTCTGAGCCCCAGACTGGAG /	12f
	GGGGTGGCAGCTTCGGGGACAATC /AGGGAAAAGGAAGGGAGGAGAAAT	12F04/R04

RESULTS

Thirty-nine patients were enrolled in this study, 30 (77%) female and nine (23%) male, from 36 different families. The mean age at onset of the disease was 6 years (2-13 years). Thirty-four (87%) of the 39 patients had onset of the disease in the first decade of life. Age of onset was defined as the age that parents noticed the first motor difficulties. The first sign of the disease were related to a proximal

muscular involvement, including: frequent falls (22 patients), difficulty climbing stairs (12 patients) and difficulty to rise from the floor (2 patients). In three patients, the first sign of the disease was a tiptoe walking. All patients showed normal milestones and achievement of independent ambulation and showed elevated CK levels. Table 3.

Table 3: Clinical and laboratorial data from 39 patients enrolled in the study

Cases	Sex/age	Age at onset (years)	First symptom	CK (X normal)	LoA (years)	Subtype of LGMD
1 A.C.S	F/15	4	Frequent falls	40x	10	Sarcoglycanopaty
2 C.S.S	F/11	5	Difficulty to rise from the floor	10x	No	Sarcoglycanopaty
3 A.P.P	F/19	8	Difficulty in climbing stairs	35x	14	Sarcoglycanopaty
4 A.M.F	F/10	6	Frequent falls	20x	No	Sarcoglycanopaty
5 D.M.J	F/18	5	Frequent falls	10x	No	Sarcoglycanopaty
6 F.A.M	F/14	6	Frequent falls	45x	No	Sarcoglycanopaty
7 T.S.P	F/11	10	Difficulty in climbing stairs	20x	No	Sarcoglycanopaty
8 T.T.S	F/13	6	Frequent falls	8x	No	Sarcoglycanopaty
9 T.T.B	F/13	4	Frequent falls	25x	8	Sarcoglycanopaty
10 V.B.C	M/18	4	Difficulty in climbing stairs	15x	No	Sarcoglycanopaty
11 W.B.S	M/21	8	Difficulty in climbing stairs	35x	14	Sarcoglycanopaty
12 O.S.S	M/11	6	Frequent falls	60x	11	Sarcoglycanopaty
13 E.F.S	F/13	9	Difficulty in climbing stairs	60x	No	Sarcoglycanopaty
14 T.A.S	F/12	4	Frequent falls	40x	No	Sarcoglycanopaty
15 F.L.F	F/13	7	Difficulty in climbing stairs	50x	12	Sarcoglycanopaty
16 R.M.F	F/12	3	Frequent falls	8,5x	No	Dysferlinopathy
17 Y.R.M	F/20	13	Frequent falls	56x	No	Dysferlinopathy
18 C.G.C	F/21	12	Difficulty in climbing stairs	57x	No	Dysferlinopathy
19 V.C.S	F/11	8	Frequent falls	46x	No	Dysferlinopathy
20 L.S.S	F/15	5	Frequent falls	25x	No	Dysferlinopathy
21 G.M.S	F/16	12	Frequent falls	40x	No	Calpainopathy
22 G.M.L	F/16	12	Tiptoe walking	40x	No	Calpainopathy
23 D.B.S	F/16	9	Difficulty in climbing stairs	10x	No	Calpainopathy

Cases	Sex/age	Age at onset (years)	First symptom	CK (X normal)	LoA (years)	Subtype of LGMD
24 L.C.P	F/7	3	Frequent falls	4x	No	Calpainopathy
25 J.P.S	F/17	4	Frequent falls	3x	No	Calpainopathy
26 L.B.W	F/8	2	Difficulty to rise from the floor	30x	7	FKRP related
27 W.S.O	M/12	8	Difficulty in climbing stairs	22x	9	FKRP related
28 A.A.L	M/19	3	Tiptoe walking	14x	No	EDMD
29 M.H.C	M/13	3	Frequent falls	2x	No	EDMD
30 M.N.P	M/18	3	Frequent falls	1,5x	No	Laminopathy
31J.A.S	F/17	2	Tiptoe walking	1,5x	No	Laminopathy
32 J.S.M	F/13	10	Difficulty in climbing stairs	2x	No	Caveolinopathy
33 B.S.N	F/16	8	Frequent falls	60x	12	LGMD ?
34 B.V.S	F/10	5	Frequent falls	50x	No	LGMD ?
35 G.H.G	M/10	2	Difficulty in climbing stairs	6,5x	No	LGMD ?
36 H.B.M	F/10	6	Frequent falls	8x	No	LGMD ?
37 L.R.C	F/17	8	Difficulty in climbing stairs	5x	No	LGMD ?
38 N.M.C	F/18	9	Frequent falls	60x	No	LGMD ?
39 M.S.V	F/19	11	Frequent falls	45x	No	LGMD ?

Among the 39 patients enrolled, 34 were defined with a limb-girdle muscular dystrophy (LGMD). All of them with a recessive form. Four patients (4/39) received diagnosis of Emery–Dreifuss muscular dystrophy (EDMD). In two, a total deficiency of emerin labeling in the nuclear membrane was detected by immunofluorescence technic, and in other two the genetic analysis identified a mutation in *LMNA* gene. One patient was diagnosed with a caveolinopathy by muscle biopsy that showed a dystrophic pattern with partial reduction of caveolin in imunohistoquemistry technic. This 5 patients were included in the total group because their mode of presentation overlapped with limb-girdle syndrome in children, but they were excluded from the general classification for calculating frequencies. Of the 34 LGMD patients, 15 (44%) were classified as Sarcoglycanopathies (LGMD Sarcoglycan-related); Five (15%), as Calpainopathy (LGMD Calpain3-related); Five (15%) as Dysferlinopathy (LGMD Dysferlin-related) and two (6%) as LGMD FKRP-related. In 7/34 patients (21%) it was not possible to define the specific subtype, despite the techniques used. Diagram 1.

Diagram 1: Frequency of LGMD in 34 children enrolled in the study

Clinical and histological data

SARCOGLYCANOPATHY (LGMD SARCOGLYCAN-RELATED)

Fifteen (three males and 12 females) were diagnosed with sarcoglycanopathy based on the results found in the muscle biopsy and immunohistochemical study. Only 13 of these patients underwent muscle biopsy, since, when there were sibling pairs, the biopsy was performed on only one. All patients in this subgroup had disease onset in the first decade of life, which ranged from four to 10 years with a median of six years; six (40%) showed their first symptoms by the age of five. Consanguinity between the parents was present in five (33.3%) suggesting a recessive pattern of inheritance, while in 10 patients (66.6%) the condition was sporadic. Signs and symptoms at clinical presentation included: frequent falls (eight patients), difficulty climbing stairs (six patients) and difficulty getting up from the floor (one patient). The pattern of weakness was predominantly proximal, with the lower extremities being affected earlier and more severely than the upper extremities in 11 patients. It was not possible to detect, through clinical history, the latency time between the age at which weakness in the lower limbs manifested and the age at which weakness in the upper limbs was noted. Facial muscles were preserved in all patients. Some semiological signs were common in these patients: winging of scapula in eight (53.3%), calf hypertrophy in six (40%), and scoliosis in four (26.6%). Six patients lost their ability to walk during the course of the disease, at the mean age of 11. The other nine patients continued to walk during the last follow-up visit, but many had significant difficulties, for example, being unable to get up from a chair or the floor without help. No patient had evidence of cognitive impairment. All patients in this group had undergone ENMG, which was compatible with a myopathic pattern. CK levels, also obtained in all patients, were elevated (10 to 60 x above normal) in the initial stages of the disease. Complete cardiological evaluation including physical examination, electrocardiogram, chest x-ray and echocardiogram was performed in all patients. In two patients, cardiomyopathy with systolic dysfunction and ejection fraction of 41 and 53%, respectively, was demonstrated. Both patients remain in cardiological follow-up. Pulmonar function test performed on all patients identified: mild restrictive disorder in one case and moderate restrictive disorder in two. These patients are undergoing respiratory physiotherapy using home ventilatory support.

The muscle biopsy performed in 13 of the 15 patients in this group showed a moderate to severe dystrophic appearance, characterized by significant variability in fiber caliber, moderate to intense increase in perimysial and/or endomysial connective tissue, nuclear centralization and necrosis with macrophages and signs of regeneration. Other findings included splitting (seven patients), hypercontracted fibers (four patients) and in one patient a torn red fiber was observed. immunohistochemical showed normal staining for merosin, dysferlin and caveolin around the fiber membrane in all cases. Concomitant dystrophin reduction was seen in one patient. In four patients there were a total absence of labeling only in α-SG and a partial reduction in the labeling of the others. In one of the 13 patients, γ-SG was completely deficient in all fibers with a partial reduction in other sarcoglycans. In other cases, there was a reduction or absence of labeling in two or more of the four sarcoglycans (eight cases). (Figure 1 and 2).

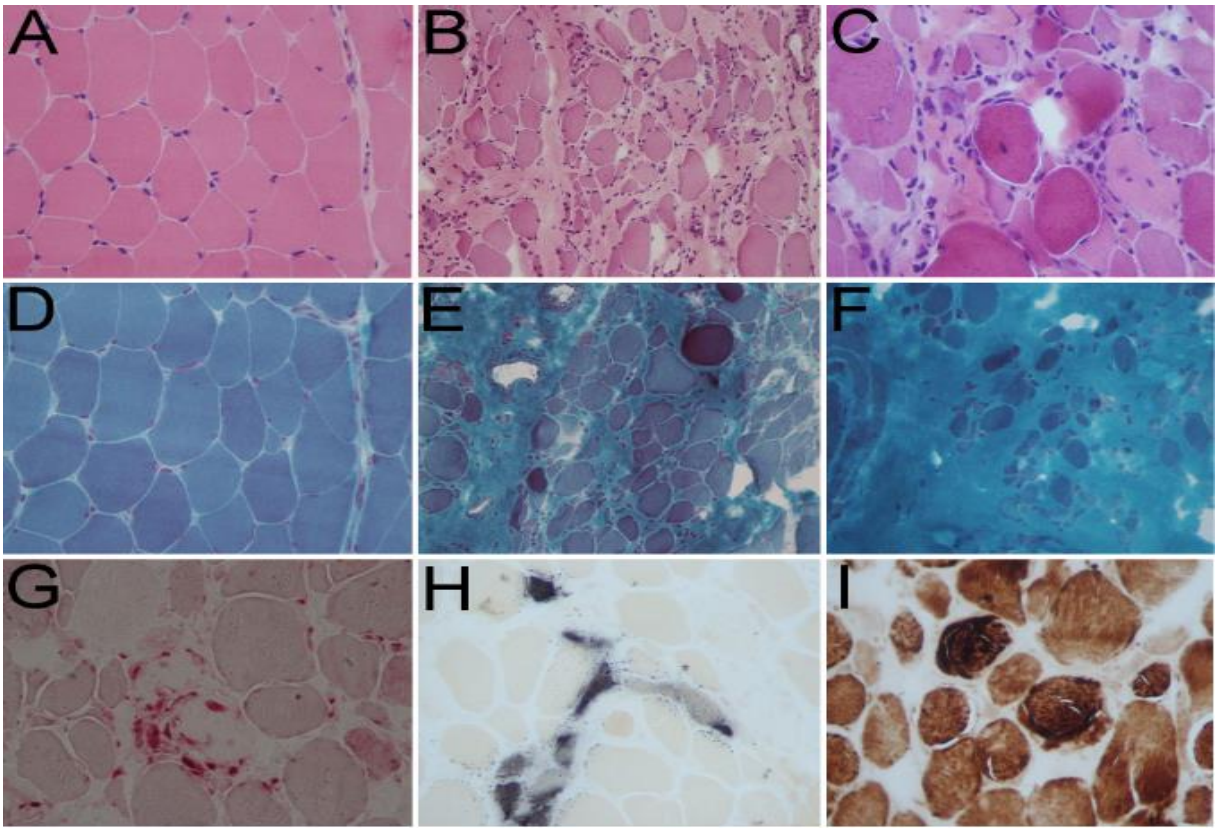

Figure 1: Normal histological pattern by HE and GO techniques (A and D) and dystrophic pattern in patients with sarcoglycanopathies (B, C, E, F, G, H and I).

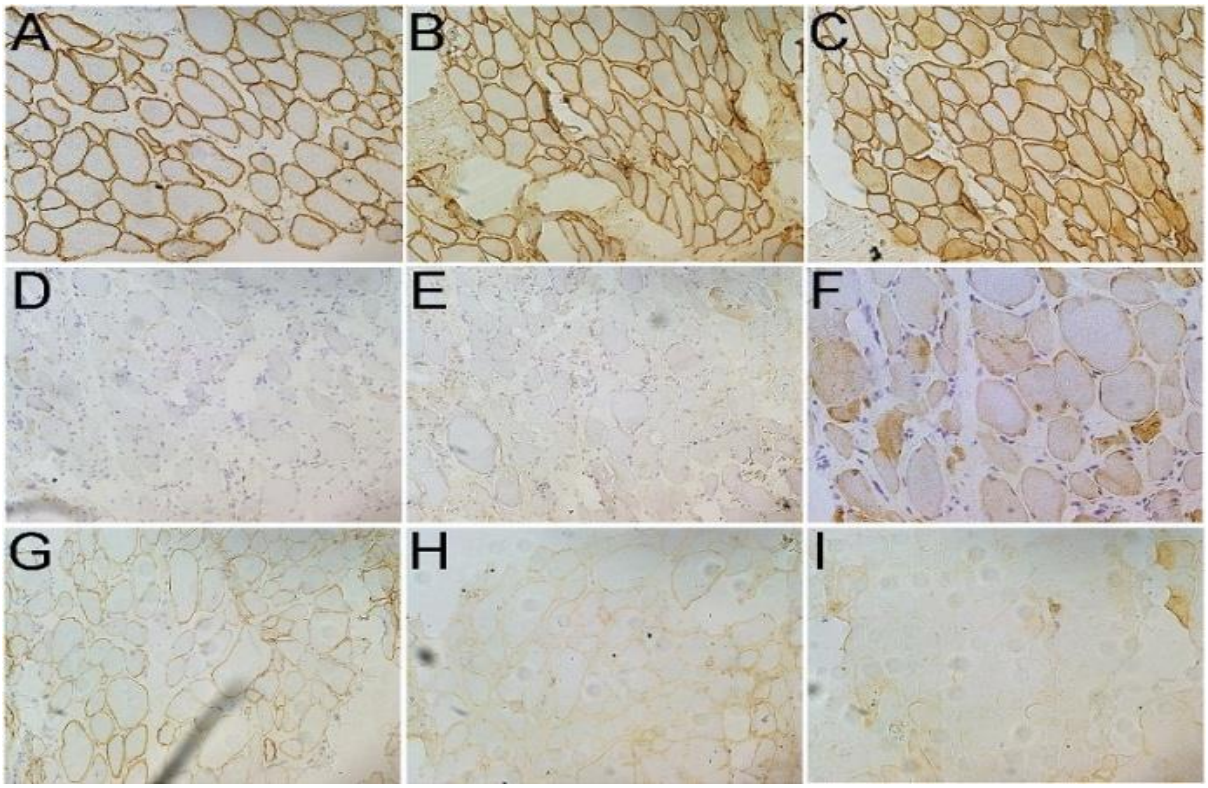

Figure 2: Muscle biopsy of patients with sarcoglycanopathies showing normal α, β and γ-SG immunostaining around the fibers (A-C) and reduced or absent labeling (D, E, F, G, H and I).

DYSFELINOPATHIES

Five patients, all female, were diagnosed with LGMD due to dysferlin deficiency demonstrated through abnormal immunostaining of the dysferlin protein in the muscle. The age at onset of the disease ranged from three to 13 years, with a median of 8 years. None of the cases had consanguinity or family history. The initial symptoms were frequent falls (four cases) or difficulty climbing stairs (one case). In all patients, weakness started in the proximal region of the lower limbs. CK levels were markedly elevated in all patients in the early phase of the disease (30-55 X normal value). Regarding trophism, three patients presented evident atrophy of the muscles of the posterior compartment of the legs and two patients also presented proximal atrophy of the biceps brachii. All had incipient retractions in the Achilles tendons. During follow-up, no patient lost walking and the disease progressed slowly. Cardiac evaluation and pulmonary function test were normal in all patients. Histologically, muscle biopsy showed a mild dystrophic pattern in all cases and inflammation was found in only one patient. immunohistochemical showed partial or absent labeling of dysferlin by immunoperoxidase or immunofluorescence techniques.

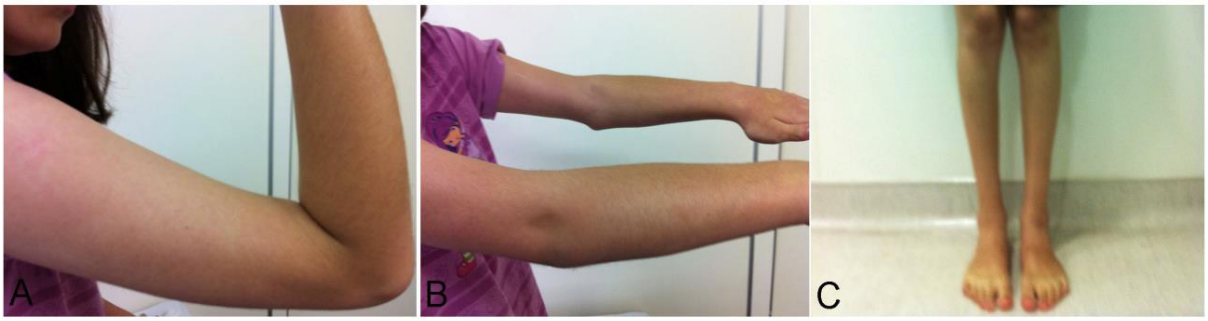

Figure 3: Patient with dysferlinopathy showing atrophy of the biceps brachii muscles (A), weakness of the upper limbs (B) and distal atrophy of the lower limbs (C).

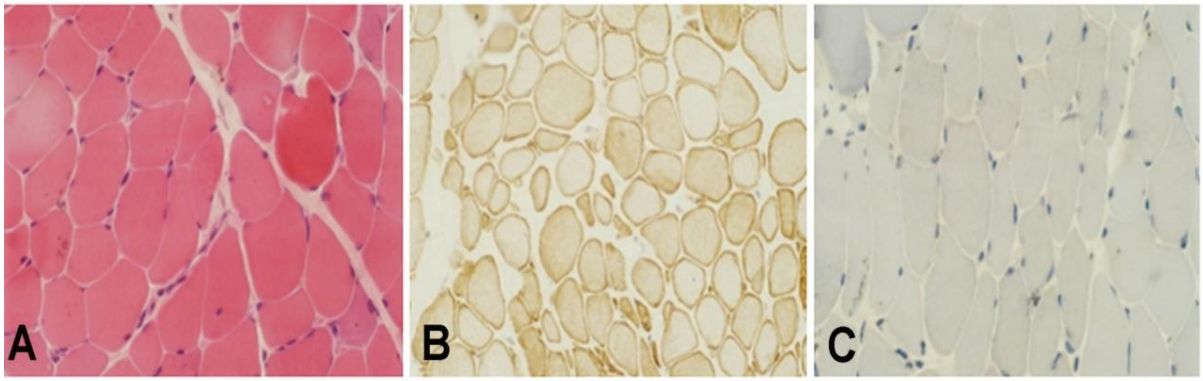

Figure 4: Muscle biopsy from a patient with disferlinopathy showing a mild dystrophic pattern using the HE technique (A) and absence of staining around the fibers on immunohistochemistry (C), compared to the normal pattern (B).

CALPAINOPATHIES

Five patients, all female, were diagnosed with calpainopathy, through the identification of pathogenic mutations in the CAPN3 gene. These patients had an age at onset of the disease between three and 12 years, with a median of nine years, with the onset of weakness in the lower limbs, manifested through frequent falls in three cases, difficulty climbing stairs in one case, altered gait in one case. The presence of Achilles retractions was found in four of the five patients and winging of scapula was found in three. CK levels were high in all cases, and in two twin sisters, levels reached 40 times the normal value. The disease showed a pattern of slow progression in all patients with the exception of one case, which after five years of progression of the condition already shows signs of imminent loss of walking. Pulmonary function tests and cardiological evaluation were normal in all patients throughout the follow-up. Four of the five patients underwent muscle biopsy, which showed a moderate dystrophic pattern in all cases. Splitting and lobulated fibers occurred in two patients and in one patient, large areas of minicore-type focal defects were found in the NADH technique. No inflammation was found in any of the biopsies of patients in this group. The immunohistochemical study showed normal staining for dystrophin, dysferlin, sarcoglycans, merosin and caveolin (Figure 5 and 6).

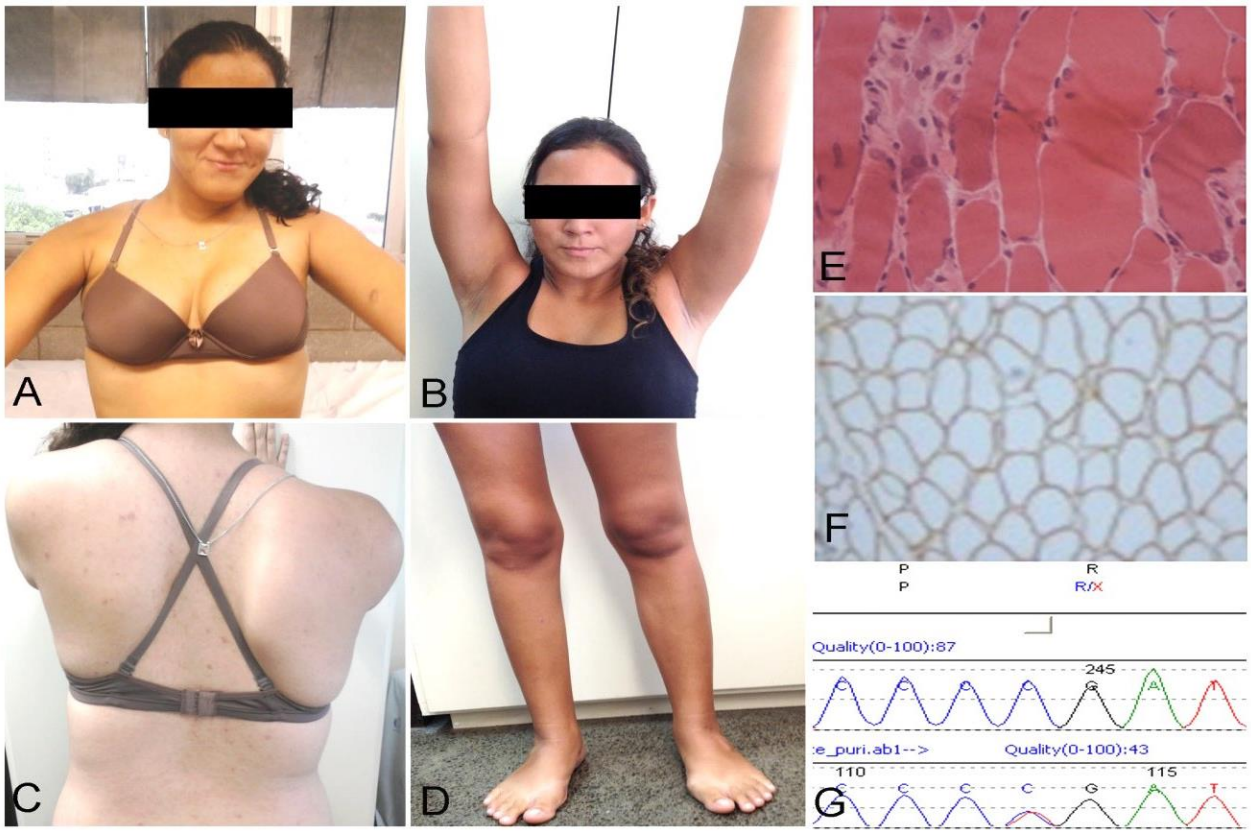

Figure 5: Twin sisters with calpainopathy,, showing intrafamilial variability. In A proximal weakness of the upper limbs; in B, strength preserved; in C, winging scapula; in D, atrophy symmetry. In E, muscle biopsy showing a moderate dystrophic pattern and in F, normal immunohistochemistry for dysferlin. Pathogenic mutation in the CAPN3 gene (G).

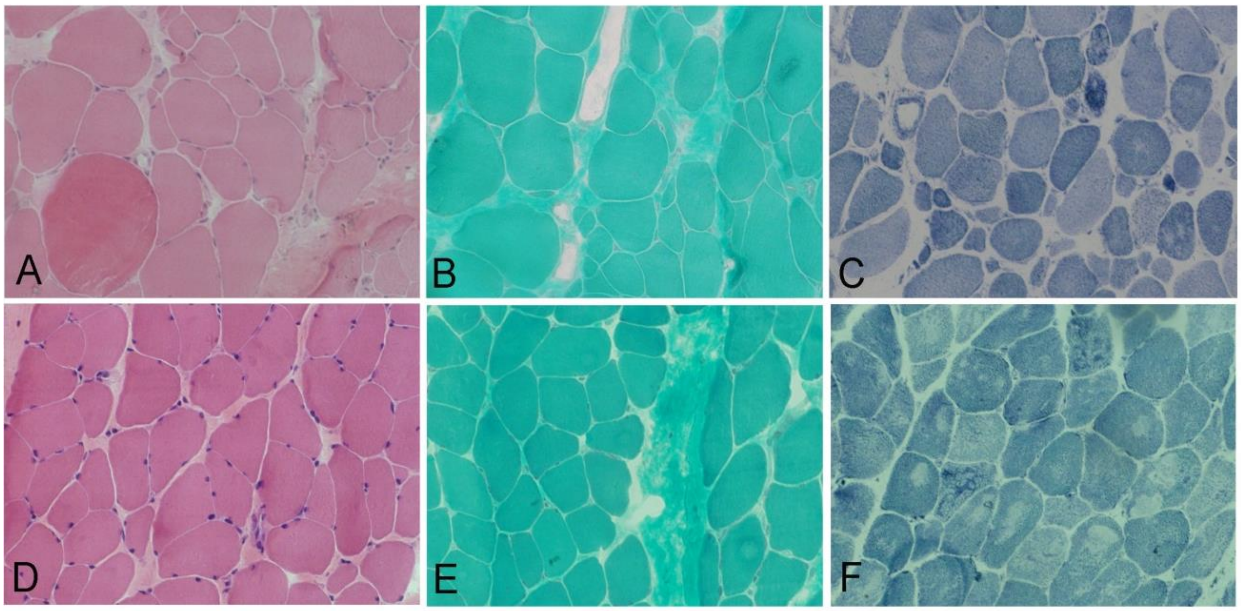

Figure 6: Histological aspects of biopsies from patients with calpainopathy: moderate dystrophic pattern in HE and GO (A, B, D, E); presence of lobulated fibers (C), and large amounts of minicore focal defects (F) using the NADH technique.

FKRP-RELATED

Two patients (6% of the total) were classified in the subtype LGMD FKRP-related through the identification of a mutation in the FKRP gene. One patient presented with weakness predominantly in the lower limbs, characterized by difficulty in getting up from the floor since the age of two years and rapidly progressive evolution with loss of gait at the age of seven. Physical examination revealed calf hypertrophy and Achilles retractions. The CK level was quite high (30X the normal value). Cardiological and respiratory evaluation was normal (Figure 7 and 8). The other patient had his illness start at eight years of age with difficulty climbing stairs. There was no calf hypertrophy, scoliosis or winging scapula. There were Achilles, popliteal and elbow retractions. The evolution was rapid, with loss of capacity to walk one year after the onset of the condition. The CK level was also elevated (20X normal). Cardiological and respiratory evaluation showed no changes.

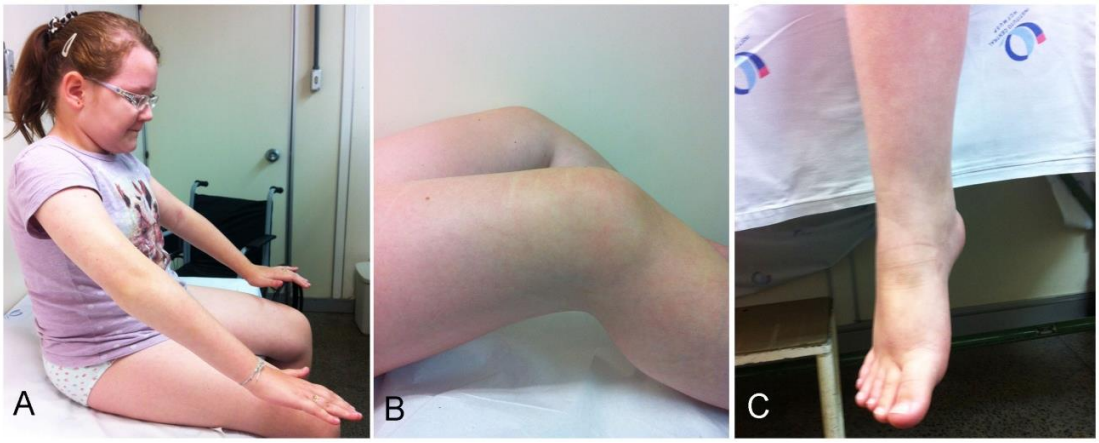

Figure 7: Patient with a mutation in the FKRP gene presenting weakness in the upper limbs (A) and retractions in the popliteal region (B) and Achilles tendon (C)

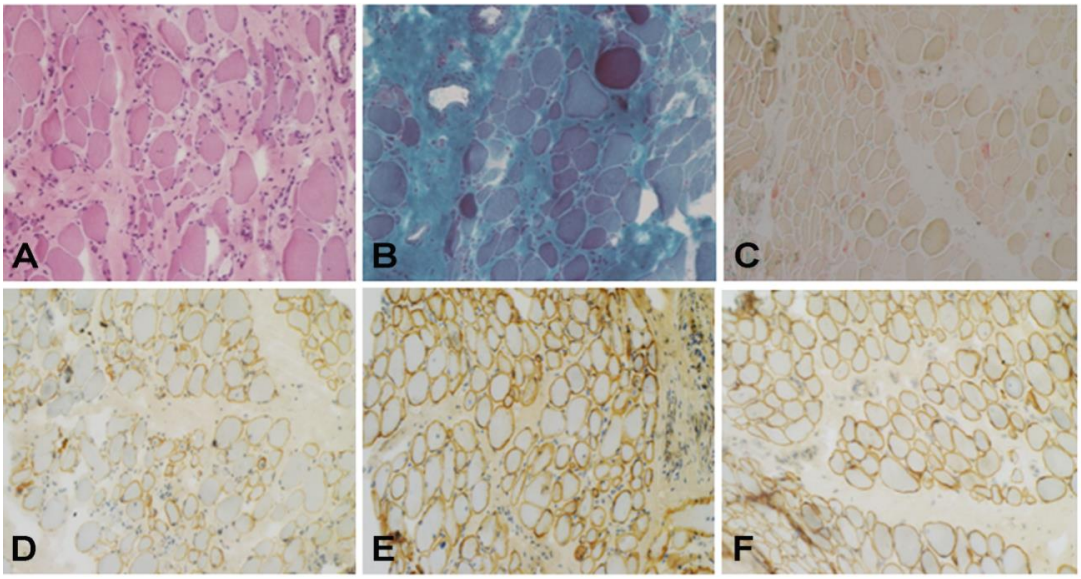

Figure 8: Histological aspects in the muscle biopsy of a patient with FKRP mutation. Moderate dystrophic pattern (A-C) and immunohistochemistry showing partial deficiency of merosin labeling (D) with normal labeling for caveolin (E) and β-sarcoglycan (F).

LAMINOPATHY

The initial symptoms of patients in this group appeared between two and three years of age and the signs presented were frequent falls and tiptoe walking. The progression of weakness was mild in one case and moderate in the other, but neither case had lost walking until the last follow-up visit. The first case is a boy (Figure 9). The disease began with falls at three years of age, associated with retractions predominantly in the Achilles tendons, with tenotomy of these tendons being performed bilaterally at nine years of age. Muscle strength remained stable until the current age, being graded between IV and V in the four limbs. The family history showed five paternal relatives with a history of heart disease (arrhythmias, sudden death). Cardiological evaluation with ECG, ECHO and Holter did not demonstrate any abnormalities to date. Similarly, the other patient, a girl, presented the condition early at two years of age with digitigrade gait and early-onset retractions in the Achilles tendons. However, disease progression in this patient was faster. Cardiological evaluation and pulmonary function test are normal. There was no case of heart disease or muscle weakness in the family. In both patients, CK levels were slightly elevated (1.5 times the normal value).

MOLECULAR ASPECTS

The diagnosis of this form was made by finding mutations in the LMNA gene. Two different mutations were identified. A new heterozygous mutation was found in exon 1 in one case and a heterozygous mutation was identified in exon 4 in another, that has already been described previously.

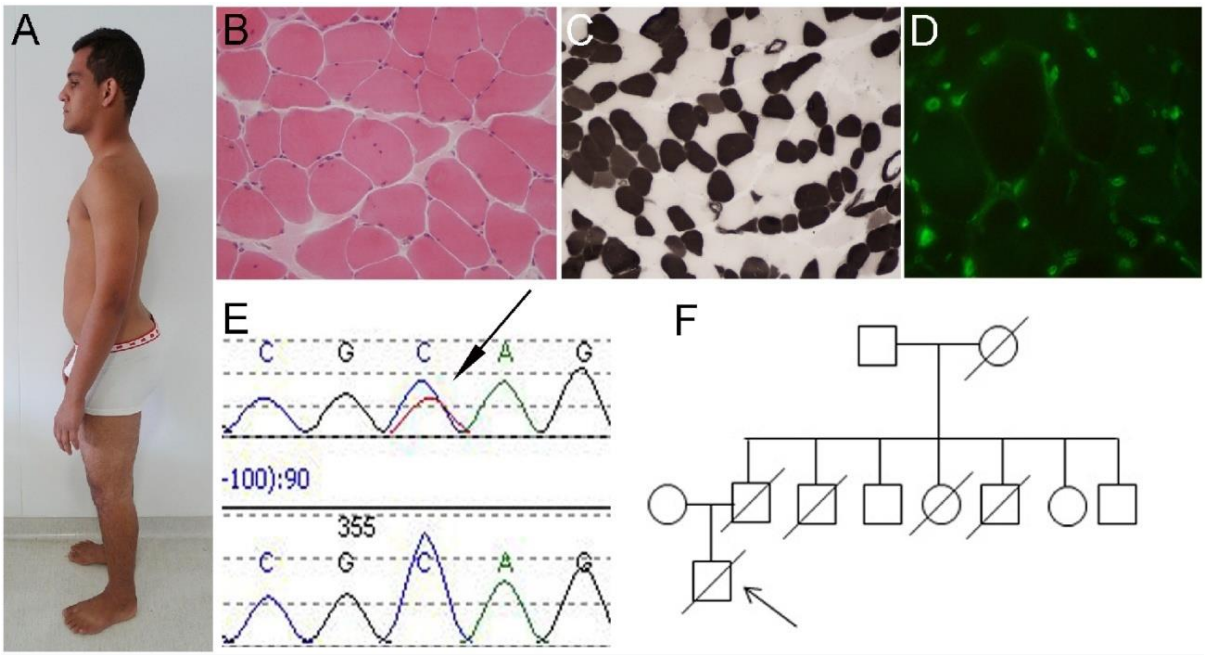

Figure 9. Patient with mutation in LMNA gene (A). In B, level dystrophic pattern using the HE technique; in C, ATPase 4.3 showing preserved mosaic; in D, normal labeling for emerin in the IMF technique; in E, heterozygous mutation in exon 1 of the LMNA gene, and in F, family pedigree.

CAVEOLINOPATHY

One patient presented with a condition that began in late childhood, characterized by weakness predominantly in the lower limbs associated with incipient retractions in the Achilles tendons. The CK level was found to be slightly elevated and the muscle biopsy showed a mild dystrophic pattern with partial reduction of caveolin labeling in the muscle and normal labeling of other proteins. The cardiological and respiratory evaluation was normal and the patient is evolving in a stable manner.

PHENOTYPES OF LIMB-GIRDLE MUSCULAR DYSTROPHIES WITHOUT DEFINED SUBTYPE

In all patients in this group, muscle biopsy showed a dystrophic pattern and immunohistochemistry showed normal staining for proteins dystrophin, dysferlin, emerin, caveolin and sarcoglycans. Patients were screened for a point mutation in the FKRP gene, which was negative. The search for mutations in the exons studied in the CAPN3 gene was also not informative. Some patients, phenotypically, resemble certain forms of limb-girdle dystrophy, but immunohistochemical or molecular study has not yet been confirmed. One patient presents a phenotype very similar to Emery-Dreifuss dystrophy with retractions in the neck, elbows and Achilles tendon. The immunohistochemistry study showed normal labeling of the emerin and lamin A/C proteins around the nuclear membrane and this patient is completing of the study to research mutations in the LMNA gene.

All patients in this group present a slowly progressive course of the disease with the exception of one patient who developed loss of walking four years after the onset of symptoms, which occurred at 12 years of age. Finally, one patient presents in muscle biopsy, in addition to the dystrophic pattern, intracytoplasmic aggregates that resemble myofibrillar myopathy. All patients had a normal cardiological evaluation so far. The pulmonary function test was performed at least once in each patient after the beginning of follow-up and only one patient presented a restrictive respiratory disorder with reduced FVC. This patient is undergoing respiratory rehabilitation.

Discussion

This was the first Brazilian clinical and frequency study on LGMD in an exclusively pediatric population followed at a center specialized in neuromuscular diseases.

Since the proposal of new nomenclature and classification of LGMD in 1995[12], some changes in the frequency of the different forms of LGMD have already occurred, particularly since 2001, when it was evidenced that changes in the enzymes responsible for the glycosylation of alpha-dystroglycan may also be responsible for forms of the disease[16-18]. Until this time, the LGMD2A and LGMD2B forms caused by mutations in the CAPN3 and DYSF genes, respectively, were the most commonly found around the world and also in Brazil[15]. From 2001 onwards, with the identification of mutations in the FKRP gene, initially associated with a form of CMD[16], and soon after with a condition of limb-girdle dystrophy[17], the prevalence changed, finding a high frequency of limb-girdle forms caused by a mutation in this gene, particularly in Eastern European countries, where it began to rival previously known forms[18-20]. After

FKRP, mutations in other genes responsible for glycosylation of alpha-dystroglycan were identified leading to LGMD subtypes, but at a lower frequency than that caused by the mutation in the FKRP gene. In 2010, with the identification of mutations in the anoctamine-5 gene, a new change occurred in the frequency and prevalence of these diseases, with a large number of cases of patients with limb-girdle dystrophy due to a mutation in this gene, being diagnosed in adults, mainly in northern European countries[21,22].

Our study enrolled 39 children, from different regions of Brazil, the majority from the State of São Paulo, and it was not possible to assess, epidemiologically, whether there is a predominance of a certain form by region. Both sexes were affected, but a higher frequency in girls than in boys, which is not observed in other studies. It must have been an occasional find, since the autosomal dominant or recessive inheritance pattern does not favor one of the sexes. There are authors who consider that, in some series, an erroneous inclusion of boys with Becker Muscular Dystrophy (DMB), who also show a limb-girdle pattern of weakness and a dystrophic pattern on muscle biopsy, with partial reduction in dystrophin in the muscle, could be judged as resulting from a primary defect[3]. We believe that this confusion could only occur when there is no access to molecular diagnosis or in cases of DMB in which the mutation is not found by molecular test. On the other hand, it is possible that patients with a phenotype and evolution similar to Duchenne/Becker dystrophy who were not referred to a specialized service, are misdiagnosed, for example, in the case of sarcoglycanopathies, and mistakenly followed up as cases of dystrophinopathy, which is the most common form of dystrophy in boys.

In our study, in eight cases (20% of the total) there was a history of consanguinity suggesting a recessive pattern of inheritance, but the majority of cases were considered sporadic. It has already been demonstrated that in Caucasian populations, autosomal dominant forms are responsible for approximately 14% of cases, recessive forms for 34% and in the majority (52% of cases) there is no family history[11].

Sarcoglycanopathies constituted 44% of cases of LGMD in the present study, being the most commonly found form. Moore et al., in a clinical and histopathological study in the United States, found a 15% frequency of sarcoglycanopathies, being the second most frequent form after dysferlinopathy[23]. In other studies, including in Brazil, it was demonstrated that sarcoglycanopathy is the most common form, particularly when we analyze the more severe forms of the disease with onset in childhood[24,25].

All patients in the sarcoglycanopathies subgroup had disease onset in the first decade of life, with ages ranging from four to 10 years (median of six years). The six patients, who lost their ability to walk, start symptoms between four and eight years of age. They lost ambulation in a time between four to six years after first symptom. These patients presented a high level of CK (25-60X normal) and a moderate or severe dystrophic pattern in muscle biopsy, data that seem to suggest a faster evolution in association with the age of onset earlier.

Some studies have demonstrated that α-sarcoglycan is a subunit of the sarcoglycan

complex most frequently reduced in primary sarcoglycanopathy[26]. Other studies show greater variability in the expression pattern, from the total absence of the entire sarcoglycan complex to the preservation of only γ-sarcoglycan with a similar partial reduction of α- and ß-sarcoglycan[27]. In the present study, deficiency of two or more of the four sarcoglycans was the most common pattern found (eight of the 13 patients who had a muscle biopsy or 61.5%). The total absence of α-sarcoglycan with partial reduction of the others was seen in four patients; in one patient there was a total absence of γ-sarcoglycan with a partial reduction of the others, and it was not possible to identify through the immunohistochemical study which sarcoglycan was primarily affected. Some studies sugest that if a patient presents an immunohistochemical study showing deficiency of all four sarcoglycans, it is evidence that is an α-sarcoglycanopathy[27-29]. However, a pathogenic mutation in the SGCG gene has already been found, confirming a case of γ-sarcoglycanopathy, with the absence of all sarcoglycans in the immunohistochemical study[29]. Consequently, since in muscle biopsy with immunohistochemical study it can be difficult to define which sarcoglycan is initially affected, demonstrating the mutation in one of the sarcoglycan genes is necessary to define where the primary defect is located.

The second most common form found in our study was a calpainopathy, diagnosed in five patients, or 15% of the total, through the identification of mutations in the CAPN3 gene.

Calpainopathy still appears to be the most frequent form of LGMD in different populations[30-32]. In Brazil it corresponds to around 30% of known cases[31]. It was the first described form of LGMD caused by the deficiency of a non-structural protein, the enzyme calpain 3, which is involved in the process of muscle contraction, as it strongly binds to titin. In a study carried out by a European consortium, in which clinical information, when available, and blood samples from 484 patients from different demographic regions were analyzed to search for mutations in the CAPN3 gene, this resulted positive in approximately 50% of the reported cases[32]. In this same study, it was observed that, when specific clinical criteria are used, the clinical correlation with the finding of mutation reaches 80% of cases. In our study, it was not possible to demonstrate calpain deficiency by Western-Blot in clinically suspected patients, due to technical difficulties. The suspected cases were subjected to research for mutations in exons 1, 2, 4, 5, 11 and 22 of the CAPN3 gene, in which more than 80% of the mutations already described are found[30,31]. In our study, five different mutations were identified in five patients, all previously described. Three patients presented a compound heterozygous mutation and in two patients only one mutation in one allele was identified in the trained exons. All patients in this group were female. Contradictorily, in recent screening identifying muscle calpain deficiency in Italian patients, there were significantly more affected men (43) than women (23)[32].

The age at which symptoms begin varies from three to 12 years, with a median of 9 years. From a clinical point of view, our patients demonstrated a classic phenotype characterized by muscle weakness with symmetrical

involvement in the pelvic and shoulder girdles. In four of the five patients, the most prominent findings were winging scapula and Achilles retractions. CK levels at the onset of the condition varied between 3 and 40 times the normal value. The progression of weakness was shown to be slow in four of the five patients and moderately in one patient, with no patient having lost walking until the last evaluation. There was no clear association between age of onset and clinical course. Cardiological and respiratory assessments were normal in all cases.

Two twin patients in which one of the most recurrent mutations in the Brazilian population, R110X in exon 2, was detected, presented slightly different clinical symptoms and evolution. Although the onset of symptoms in both was at 12 years of age, the progression was faster in one, demonstrating that also in calpainopathy, as well as in sarcoglycanopathies, there is intrafamilial variability, which had already been demonstrated in other studies[36]. One of the twins initially presented with proximal weakness predominantly in the lower limbs, of a progressive nature, leading to significant difficulty in walking. Physical examination revealed winging scapulae and calf hypertrophy, associated with Achilles retractions. His sister presents a lighter condition, with discreet Achilles retractions, winging scapulae, but without calf hypertrophy. Her muscular strength appears slightly reduced. Interestingly, both patients showed, in serial blood counts, persistent eosinophilia, which has already been reported in patients with mutations in the CAPN3 gene and eosinophilic myositis[37].

In muscle biopsies from cases of calpainopathy, in addition to the characteristic dystrophic pattern, the presence of lobulated fibers was found in two cases. Although this represents a non-specific abnormality observed in different neuromuscular diseases, several studies suggest that its presence is frequent in calpainopaty, when compared to other forms of LGMD, and is therefore a finding that can guide molecular studies[38].

Five patients (15%) were classified dysferlinopathy, which together with calpainopathy, was the second most common form in our study. Previous studies in Brazil had already shown that dysferlinopathy is as common as calpainopathy and presents a slightly more benign phenotype[13]. In Italy, it is the second most frequent after LGMD calpain3-related[39].

All LGMD dysferlin-related cases in our study were female patients. The median age of onset of symptoms in this group was eight years, similar to that of patients with LGMD calpain3-related, but with a later onset compared to the age of onset of sarcoglycanopathies. Previous work had already demonstrated that the disease can have a variable onset, from early in childhood to cases of patients who remain asymptomatic until 60-70 years of age, with the average age of onset, when analyzing the forms of onset in children and adults, it is about 20 years[40-42].

The predominant phenotype in this form was proximal weakness predominantly in the lower limbs, associated with distal atrophy, which was observed in three patients. This clinical pattern, with involvement of the muscles of the posterior compartment of the legs, is the most commonly described in patients with LGMD dysferlin-related, and is an important sign when considering the

diagnosis[41,42]. All cases showed a slow evolution, with no loss of gait occurring in this group of patients. Studies show that dysferlinopathy presents a slowly progressive progression with an average age for needing a wheelchair at 38 years[41].

The CK level ranged from 1600-10998 U/L, with an average of 7400 U/L. Elevated CK levels are not specific to LGMD dysferlin-related, but are generally seen in the early stages of the disease and can be as high as 50-100 times the normal value. With evolution, CK levels tend to fall[42].

A mild dystrophic pattern was found in the muscle biopsy, but the characteristic endomysial inflammation that can be found in this form of the disease was observed in only one patient. Studies suggest that this inflammatory aspect may be minimal in the early stages of the disease, contrary to the high levels of CK, which may justify the absence of this finding in our patients[42]. In these cases, the pattern of evolution has been slow without respiratory or cardiac compromise until the date of the last evaluation.

Since its original description, it has become evident that LGMD FKRP-related is one of the most common forms of LGMD in adult populations in the United States, Denmark, and Germany, possibly rivaling calpainopathy and dysferlinopathy in frequency[43,44]. In Brazil it appears to be a less frequent form, but the real frequency still needs to be estimated. In our study, two patients (6% of the total) were identified with two different mutations in the FKRP gene, making LGMD FKRP-related the third most common form of recessively inherited limb-girdle dystrophy. In an Italian and Polish descent patient, the homozygous missense mutation C826A was identified, leading to the change of amino acid p.L276I in the FKRP gene, which is the most common mutation found in European populations, unlike what occurs in Asian populations where pathogenic mutations appear to be sporadic[45].

There is great clinical variability associated with this mutation, from mild forms with late onset to Duchenne-like forms[46]. However, this mutation, when homozygous, generally causes a mild phenotype with late onset and loss of gait in advanced age; in contrast, in this patient, the first symptoms appeared at two years of age and the course was rapidly progressive, Duchenne-like, with the need for a wheelchair due to loss of walking at seven years of age. Calf hypertrophy was present as well as Achilles retractions. CK levels were quite high and muscle biopsy showed a moderate dystrophic pattern with immunohistochemistry showing partial deficiency of merosin labeling and normal labeling of other proteins. In another patient, the mutation was only found in one allele, being characterized by a 585C>T nucleotide exchange. We were unable to identify a second mutation in this patient, which strongly suggests that mutations may exist outside the FKRP coding region. Therefore, we do not have confirmation that this patient is actually affected by LGMD2I; however, clinical findings suggest so and this 585C>T mutation has already been previously described in patients with LGMD FKRP-related, including in Brazil[47]. In our patients, cardiac and respiratory involvement was not demonstrated until the date of the last evaluation, although some studies show a high prevalence of this complication that may appear at more advanced ages[43-45].

Laminopathies due to mutation in the *LMNA* gene, is allelic to the autosomal dominant form of Emery-Dreifuss dystrophy. The clinical pictures of the two forms are relatively different, but there may be intermediate forms between the two[48]. Studies show that the frequency of these diseases is low[49]. In our series, two patients were found with different mutations in the LMNA gene. The patients presented a clinical picture intermediate between the two entities. These patients showed a clinical picture with a similar onset, characterized by weakness predominantly in the limb, but without humero-peroneal involvement, starting between two and three years of age, associated with early contractures in the Achilles tendon. However, the clinical progression between the two was different, being mild in one patient and moderate in the other. Muscle biopsy showed a mild dystrophic pattern in both patients and CK levels were slightly elevated. In one patient, a mutation was identified with a nucleotide insertion at position 43C>CT in exon 1 leading to a stop codon at this position p.Q15X, this being a new mutation. In the other patient, the heterozygous mutation c.745T>C in exon 4 was identified, which results in the replacement of arginine by tryptophan (p.R249W). In this case, symptoms began at two years of age, earlier than the average age of onset for patients with severe EDMD-AD (32 months) [48]. Interestingly, this same mutation was described in association with congenital muscular dystrophy due to lamin A/C deficiency, starting in the first year of life[50].

Before molecular era, time when this study was realized, it was clear that diagnosing the specific subtype of LGMD in children often was a challenge, since clinical findings overlap and the results of usual exams such as CK level and muscle histopathology are informative only in a small group of patients. To correctly identify the subtype in a child with symptoms suggestive of LGMD, a complete evaluation is essential, including family history, mode of inheritance, clinical presentation of the disease, pattern of muscle involvement, association with other clinical findings, in addition to complementary exams, particularly the CK level. Subsequently, muscle biopsy can be partially informative both through the histological aspect presented and through the immunohistochemical study and WB showing the deficiency of specific proteins involved in the different forms of LGMD. It is also important to consider the frequency of each LGMD subtype in different populations since although all forms are rare, with an estimated prevalence of 1:14500 to 1:123000, some forms have only been described in a few families or in specific regions. However, nowadays, the gold standard diagnostic method is the detection of mutations through gene analysis. Using this entire arsenal of diagnostic tools, studies show that even in completely evaluated series, an accurate diagnosis is only achieved in around 70-75% of cases[51]. The search for a specific diagnosis through the search for mutations in the different genes of the different forms of LGMD should always be aimed mainly at carrying out correct genetic counseling and recognizing possible respiratory and cardiac complications, such as respiratory failure and cardiomyopathies, especially arrhythmic ones, in which adequate management can lead to an improvement in the quality of life and survival of these patients.

Our study on LGMD in children demonstrates that if a child has a typical limb-girdle syndrome phenotype and an autosomal recessive mode of inheritance, one of the forms of sarcoglycanopathy is the most likely diagnosis, especially in the presence of calf hypertrophy, winging scapulae and/or scoliosis. Unlike the adult population, LGMD FKRP-related was not frequent in our pediatric population, but showed a severe and early-onset phenotype. LGMD calpain3-related and LGMD dysferlin-related showed a similar frequency in children, a milder phenotype, a later onset than previous forms and a slowly progressive evolution.

Conclusions

1. The present study on LGMD including only children, showed the following frequencies among the different forms: Sarcoglycanopathies (LGMD Sarcoglycan-related) 45% of cases; Calpainopathy (LGMD Calpain3-related) and Dysferlinopathy (LGMD Dysferlin-related) 15% each one LGMD FKRP-related 6%. In 21% of cases the specific subtype was not identified.

2. Clinically, Sarcoglycanopathies (LGMD Sarcoglycan-related) and LGMD FKRP-related 6% proved to be the most severe forms, with an earlier onset of the disease, generally in the first years of life, and a more aggressive course, often Duchenne-like, which can lead to loss of ambulation few years after onset of the symptoms.

3. In Sarcoglycanopathies, calf hypertrophy, scoliosis and winging of scapulae were very common.

4. In LGMD FKRP-related, the presence of calf hypertrophy is suggestive.

5. In Capainopathy and disferlinopathy, a clinical picture of milder evolution is found, generally beginning at the end of the first decade, with winging of scapulae and Achilles tendon retractions being common in LGMD Calpain3-related and a pattern with distal involvement in the lower limbs with atrophy of the muscles of the posterior compartment and discrete Achilles tendon retractions in LGMD Dysferlin-related.

6. CK levels, in general, were higher in dysferlinopathies and sarcoglycanopathies when compared to other forms. Muscle biopsy showed a more intense dystrophic pattern in sarcoglycanopathies and in cases due to mutation in FKRP gene.

7. In dysferlinopathies, the presence of inflammation was not a characteristic finding, unlike that found in adults. In Calpainopathy, the presence of lobulated fibers was the most striking finding.

LGMD FKRP-related, common in European populations, was uncommon in Brazilian children, conforming showed in the present study.

Conflicts of Interest Statement:

The authors declare that there is no conflict of interests regarding the publication of this article.

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