Arq Neuropsiquiatr 2005;63(3-B):791-800

DYSTROPHIN-GLYCOPROTEINS ASSOCIATED IN CONGENITAL MUSCULAR DYSTROPHY

Immunohistochemical analysis of 59 Brazilian cases

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ABSTRACT - The congenital muscular dystrophies (CMD) are heterogeneous muscular diseases with early and dystrophic pattern on muscle biopsy. Many different subtypes have been genetically identified and most phenotypes not yet identified belong to the merosin-positive (MP) CMD subgroup. *Objective:* To analyze the immunohistochemical expression of the main proteins of the dystrophin-glycoproteins associated complex in muscle biopsy of patients with different CMD phenotypes, for investigating a possible correlation with clinical and histopathological data. *Method:* Fifty-nine patients with CMD had clinical, histopathological and immunohistochemical data evaluated: 32 had MP-CMD, 23 CMD with merosin deficiency (MD-CMD), one Ullrich phenotype and three Walker-Warburg disease. *Results:* Dystrophin and dysferlin were normal in all; among the patients with MD-CMD, merosin deficiency was partial in nine who showed the same clinical severity as those with total deficiency; the reduced expression of α-sarcoglycan (SG) and α-dystroglycan (DG) showed statistically significant correlation with severe MD-CMD phenotype. *Conclusion:* There is a greater relationship between merosin and the former proteins; among MP-CMD patients, no remarkable immunohistochemical/phenotypical correlations were found, although the reduced expression of β-DG had showed statistically significant correlation with severe phenotype and marked fibrosis on muscular biopsy.

KEY WORDS: congenital muscular dystrophy, merosin, dystrophin-glycoproteins associated complex, sarcoglycan complex, dystroglycan complex.

Complexo distrofina-glicoproteínas associadas na distrofia muscular congênita: análise imunohistoquímica em 59 casos

RESUMO - A distrofia muscular congênita (DMC) é doença muscular heterogênea, de início precoce e padrão histopatológico de distrofia. Diversos subtipos foram geneticamente identificados e os fenótipos ainda não identificados pertencem em geral ao subgrupo de DMC merosina-positiva (MP). *Objetivo:* Analisar a expressão imuno-histoquímica das principais proteínas do complexo distrofina-glicoproteínas associadas na biópsia muscular de pacientes com diferentes fenótipos de DMC, a fim de investigar uma eventual correlação com o quadro clínico e histopatológico. *Método:* Cinqüenta e nove pacientes com DMC foram avaliados clinicamente e sua biópsia muscular, histopatologica e imuno-histoquimicamente: 32 eram MP, 23 merosina-deficiente (MD), um mostrava fenótipo Ullrich e três síndrome de Walker-Warburg. *Resultados:* Distrofina e disferlina foram normais em todos; nove pacientes MD apresentavam déficit parcial de merosina, porém com a mesma gravidade clínica daqueles com deficiência total. *Conclusão:* A hipoexpressão de α-sarcoglicana (SG) and α-distroglycan (DG) se correlacionou estatisticamente com o grave fenótipo MD, assim indicando maior correlação entre a merosina e as referidas proteínas; entre os pacientes MP, apesar da hipoexpressão de β-DG ter se correlacionado significativamente com fenótipo e histopatologia mais grave, não houve correlação clínica/imuno-histoquímica valorizável.

PALAVRAS-CHAVE: distrofia muscular congênita, merosina, complexo distrofina/glicoproteínas associadas, sarcoglicanas, distroglicanas.

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Received 6 January 2005, received in final form 8 April 2005. Accepted 27 May 2005.

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Congenital muscular dystrophy (CMD) represents a heterogeneous group of diseases characterized by early onset of hypotonia and weakness (neonatal or during the first year of life), and non specific muscular dystrophic pattern[1,2]. The disorder can be limited to the muscles or associated with the central nervous system (CNS) and/or eye abnormalities. Different specific phenotypes have been described, many of them defined on a molecular basis[3]. The most common form, CMD1A, accounting for about 40% of the cases, is due to mutations in LAMA2 gene (6 q2), which codes for α2-laminin2 (merosin)[1-4]. Other defined although rarer forms are: Ullrich CMD (COL6A1, COL6A2, COL6A3 genes, 21q22, 2q 37) with α-1, α-2 or α-3 collagen VI deficiency[4-5]; CMD with rigid spine (SPT1 gene, 1p35) with selenoprotein N 1 deficiency[6]; CMD1C (FKRP gene, 19q1) with fukutin related protein deficiency[7]; CMD1D (LARGE gene, 22q12) with acetylglucosaminyltransferase-like protein deficiency[8]; Fukuyama CMD (FCMD gene, 9q31) with fukutin deficiency[9]; muscle-eye-brain (MEB) disease (POMGnT1 gene, 1p33) with O-mannose β-1,2-N-acetylglucosaminyltransferase deficiency[10] and Walker Warburg(WW) CMD (POMT1 gene, 9q34) with deficiency of O-mannosyltransferase[11]. Cases with no identified genetic defects belong to the merosin-positive (MP) CMD subgroup. In 1998, an early onset muscular dystrophy with diaphragmatic involvement, early respiratory failure, calf or generalized muscle hypertrophy and secondary alpha2 laminin deficiency was assigned to 1q42 and named CMD 1B[12] but a specific gene or protein has not been defined yet.

The above classification of CMD forms was in part facilitated by the recent knowledge about defined or putative glycosiltransferases that interfere with the glycosilation of dystroglycan (DG) from the muscular membrane. The muscle-eye-brain forms, i.e. Fukuyama CMD, MEB disease and WW syndrome, and other forms of CMD with normal merosin or partial deficiency (CMD1B, 1C and 1D) have been associated with glycosilation defects of a-dystroglican[3,13,14] which currently represent a broad field for researchers. In spite of the continuous advances, within the MP-CMD subgroup there are some apparently specific clinical phenotypes that could represent new genetic and biochemical subtypes not yet identified.

Our objective was to analyse the expression of the components of the dystrophin-glycoproteins associated complex (DGA) in muscle biopsy of patients with different forms of CMD, in an attempt of correlating such findings with clinical and histopathologic data.

METHOD

From January 1990 to June 2002 we have followed 59 patients aged 0 to 15 years with a diagnosis of CMD based on clinical, i.e.early onset of muscle weakness and hypotonia, as well as a histopathological criteria that define dystrophic pattern on muscle biopsy, i.e. fiber size variability, endo/perimysial fibrosis and fatty infiltration (Tables 1 and 2). All patients have been examined periodically by one of us.

Muscle samples were obtained from the biceps brachial, rapidly frozen in liquid nitrogen and processed by routine histological techniques. The intensity or amount of the above histopathological changes were graded as follows: - absent; + mild; ++ moderate; +++ marked; ++++ severe and widespread.

The immunohistochemical study was performed on muscle sections by means of immunofluorescency15 or immunoperoxidase methods, using the following primary antibodies to: merosin, 80 kDa, (monoclonal, Life Technologies), diluted 1/1000; laminin α2 chain (merosin), 300 kDa (Mer 3/22B2, Novocastra), diluted 1/800; dystrophin, carboxy terminus (Novocastra, Dy8/6C5), diluted 1/1000; α-sarcoglycan (Novocastra, Ad1/20A6), diluted 1/100-200; β-sarcoglycan (Novocastra, bSarc/5B1), diluted 1/100-200; γ-sarcoglycan (Novocastra, 35DAG/21B5), diluted 1/100-200; δ-sarcoglycan (Novocastra, dSarc3/12CI), diluted 1/100-200; β-dystroglycan (Novocastra, g43DAG1/8D5), diluted 1/100-200; dysferlin (Novocastra), diluted 1/20; collagen type VI (Development studies Hybridoma Bank), diluted 1/100 e α-dystroglycan (kindly given by Dr. Stephan Kroger), diluted 1/2000. FITC-conjugated anti-mouse was used as a secondary antibody. The immunoreactivity evaluation, performed by two of the authors, followed Hayashi methodology[16]: negative (-); minimal (±), positive or normal (P) and decreased or irregular (weak).

The following clinical features were evaluated: age at onset, maximal motor ability, serum creatine kinase (CK) level, mental status and brain neuroimaging changes (Tables 1 and 2).

A statistical analysis was performed utilizing the Pearson chi-squared test17 for testing the possible association or independence between each component of the clinical, histopathological and immunohistochemical variables categories. All results were considered as mean ± standard deviation and expressed as a level of significance of 0.05 (α = 5%).

RESULTS

After clinical evaluation, immunohistochemical test for merosin and brain neuroimaging procedures, 32 patients were classified as MP, 23 as MD, one as Ullrich CMD and three as WW syndrome (Tables 1 and 2).

With regard to 32 MP patients (21 male, 11 female),12 were not symptomatic at birth and 5 nev-

er acquired independent walking (Table 1). One child presented marked cervical weakness. Three had minor mental retardation (MR), one moderate MR and two, among 22 who were submitted to magnetic resonance imaging (MRI), had focal and nonspecific white matter abnormalities. Other abnormal findings on MRI were brain cortical atrophy in two patients and cerebellar atrophy in one. CK was normal in 9 patients, 2 to 5 times up from the normal in 13, up to 5 times the normal in 7 and not available in three patients. Two children had cataracts and one had Type 1 diabetes mellitus.

All the 23 MD patients (11 male, 12 female) manifested symptoms at birth and only three acquired independent walking (that was after lost in two). One of them recovered independent walking for a period while receiving deflazacort. All patients had normal intelligence and presented white matter

Table 1. Clinical and histopathologic aspects in 32 patients with MP-CMD, one with Ullrich CMD and three with Walker-Warburg syndrome.

Case	Gender	Onset	Walking	Other findings	CK↑	MRI	Age at biopsy	Endo mysial fibrosis	Pery misial fibrosis	Size variation	Fatty tissue
1	M	NB	Supported		2x	N	1y10mo	+	++	++	++
2	M	> 6mo	+		5x	NA	14y	+	+	++	+
3	M	> 6mo	+		NA	NA	5y9mo	+	++	++	++
4	M	NB	+	Hyperlaxity	3x	BA	6y6mo	+	++	++	+
5	M	NB	+		3x	N	8y8mo	+	++	++	+
6	M	NB	Supported	Cataracts, MR	3x	N	14y2mo	+	++	++	++
7	F	NB	Supported		N	N	2y5mo	+	++	++	++
8	F	NB	+		3x	N	9y1mo	+	++	++	+++
9	F	> 6mo	Supported	Hyperlaxity	N	N	3y6mo	+	++	++	++
10	M	> 6mo	+		1,5x	N	5y6mo	+	++	++	+
11	M	NB	-		N	N	1y1mo	++++	++++	++++	++++
12	M	NB	-	MR	8x	WB	6y1mo	++++	++++	+++	+++
13	F	> 6mo	+	Hyperlaxity	N	N	14y	+	+	++	+
14	M	> 6mo	+	Rigid spine	N	NA	15y7mo	+	++	++	++
15	F	> 6mo	+		6x	V	6y3mo	+	++	++	++
16	M	NB	Supported		N	N	2y8mo	+	++	++	+
17	M	NB	Supported		NA	NA	10y	+	++	+	++
18	M	> 6mo	+		6x	NA	3y	-	+	++	+
19	F	NB	+	Hyperlaxity	7x	NA	8y3mo	+	++	+++	+++
20	M	> 6mo	+		1,5x	NA	7y	+	+	++	+
21	F	NB	+		1,5x	NA	5y5mo	+	++	+++	+
22	M	NB	+		N	N	3y7mo	+	++	++	+++
23	M	NB	+	Rigid spine	N	NA	12y	+	++	++	+
24	M	NB	+	Cataracts	3x	CeA	11y2mo	-	+	+	-
25	M	> 6mo	-		4,5x	N	1y	+	++	+	+
26	F	NB	+		4x	NA	13y5mo	+	+	++	+
27	F	> 6mo	+	MR	NA	N	2y7mo	-	+	++	-
28	M	> 6mo	+		7,5x	N	9y1mo	++	++	++	+
29	M	> 6mo	+		N	NA	11y9mo	+	++	+++	++
30	M	NB	+	Hyperlaxity	1,5x	N	3y10mo	+	++	++	++
31	F	NB	-		7x	WM	1y1mo	+	++	++	+
32	F	NB	-	Diabetes I	14x	WM	10mo	+++	+++	+++	+++
33	M	NB	-	Ullrich	N	N	9y4mo	++	+++	+++	++++
34	F	NB	-	MR	11x	Li	1y	+++	+++	+++	+++
35	F	NB	-	MR	12x	Li	2mo	+++	+++	+++	+++
36	M	NB	-	MR	N	Li	1y	-	-	+	+

M, male; F, female; mo, months; NB, newborn; MR, mental retardation; ↑, increased; MRI, magnetic resonance imaging; N, normal; NA, not available; BA, brain atrophy; WM, non specific white matter changes; V, vascular changes; CeA, cerebellar atrophy; Li, lissencephaly; y, years.

Table 2. Clinical and histopathologic aspects in 23 patients with MD-CMD.

Case	Gender	Walking	CK↑	Age at biopsy	Endo mysial fibrosis	Pery misial fibrosis	Size variation	Fatty tissue
37	F	-	NA	4y1mo	++++	+++	+++	++++
38	F	Supported	NA	1y5mo	+++	+++	+++	+++
39	F	-	27x	10y7mo	++	+++	+++	++++
40	M	-	14x	9y3mo	++	++	++	++
41	M	-	2x	3y	+++	+++	+++	+++
42	F	-	1.5x	1y4mo	+++	+++	+++	++++
43	F	-	6x	1y9mo	+++	++++	+++	+++
44	M	-	5x	2y	++++	++++	+++	++
45	M	-	13x	11mo	+++	++++	+++	+++
46	M	-	23x	1y3mo	+++	+++	+++	+++
47	F	+/lost	5x	10y5mo	++	+	++	+++
48	F	-	35x	1y2mo	+++	+++	+++	+++
49	M	-	12x	2y5mo	+++	++++	+++	++
50	M	-	3x	3y6mo	++	+++	+++	+++
51	M	-	4x	1y4mo	+++	+++	+++	+++
52	M	-	6x	5y5mo	+++	++++	+++	++
53	F	+	9x	1y10mo	++	++	+++	+
54	F	+/lost	4x	4y	+++	+++	+++	+++
55	F	-	3x	3y8mo	+++	++	+++	++
56	M	-	12x	2y8mo	+++	++	+++	+
57	F	-	7x	2y4mo	+++	+++	+++	++
58	M	-	1,5x	4y1mo	+	++	+++	++
59	F	-	1,5x	4y3mo	++	+++	+++	+++

M, male; F, female; ↑, increased; NA, not available; years; mo, months.

abnormalities on brain MRI. CK was normal in one patient, increased until 5 times in 10, above 5 times in 11 patients, and not available in one (Table 2).

The only patient with Ullrich phenotype, confirmed as UCMD by molecular analysis[18] had symptoms at birth and never acquired walking. He had normal intelligence and normal CK level.

All the 3 children with WW syndrome (1 male, 2 female) manifested symptoms at birth, were never able to walk, presented severe MR and neuronal migration defects (lissencephaly) on brain MRI. The CK level was normal in one patient and increased above 10 times in the remaining two.

Eight patients (Cases 12, 32, 34, 35, 36, 39, 40 and 55) died due to respiratory intercurrences. Three of them (Cases 34, 35, and 36) were diagnosed as WW syndrome, three (Cases 39, 40, and 55) had MD-CMD and among the two MP-CMD patients who died, one had a severe MR (Case 12) and the other had Type 1 diabetes mellitus (Case 32).

Immunohistochemical data (Table 3)

Dystrophin and dysferlin expression were normal in all patients. Among the 23 MD patients, the deficiency was partial in 9 (detected with the antibody 80 Kda in two, 300 Kda in 5, and both in two).

Concerning α-sarcoglycan (SG) and γ-SG expression, each one was reduced in 5 (15.6%) of the 32 MP patients, as well as in 18 (78.2%) and 8 (34.7%) of the 23 MD patients, respectively. The δ-SG and β-SG expression was evaluated in 31 MP and in 23 MD patients and resulted normal in all, except in one MP patient, who presented a reduced expression of β-SG.

The β-DG reaction resulted weak in 7 patients (22.5%) among 31 with MP and in 6 (26%) among 23 with MD. The a-DG reaction, evaluated in 22 MD and in 22 MP patients, resulted weak in 11 (50%) and in two (10%) patients, respectively. Additionally, in two patients with WW a weak strain was observed in each one of β-SG and γ-SG, respectively.

The reduction of the expression of a-SG and α-DG showed statistically significant correlation (Table 4) with the diagnosis of MD-CMD ($p = 0,00$ e $p = 0,003$, respectively)

Among MP-CMD patients, the reduced expression of the β-DG had statistically significant correlation with both, a severe phenotype (walking abili-

Table 3. Results of immunohistochemical analysis using different antibodies on muscular samples of 59 CMD patients.

Case	α2-LM 80 kd	α2-LM 300 kd	Col VI	DYS-C	α-SG	β-SG	γ-SG	δ-SG	α-DG	β-DG
1	P	P	NA	P	P	P	P	P	P	P
2	P	P	NA	P	P	P	P	P	P	P
3	P	P	P	P	P	P	P	P	NA	P
4	P	P	P	P	P	P	P	P	NA	P
5	P	P	P	P	P	P	P	P	P	P
6	P	P	P	P	P	P	P	P	P	P
7	P	P	NA	P	P	P	P	P	NA	P
8	P	P	NA	P	P	P	P	P	P	P
9	P	P	NA	P	P	P	P	P	P	P
10	P	P	NA	P	P	P	P	P	NA	P
11	P	P	NA	P	P	NA	P	NA	NA	NA
12	P	P	P	P	W	P	W	P	P	W
13	P	P	NA	P	P	P	P	P	P	P
14	P	P	NA	P	P	P	W	P	NA	P
15	P	P	NA	P	W	P	P	P	P	P
16	P	P	NA	P	P	P	P	P	P	P
17	P	P	P	P	P	P	W	P	P	P
18	P	P	P	P	P	P	P	P	P	P
19	P	P	P	P	P	P	P	P	P	W
20	P	P	P	P	W	W	P	P	P	W
21	P	P	NA	P	W	P	W	P	P	P
22	P	P	P	P	P	P	P	P	P	P
23	P	P	P	P	P	P	P	P	P	W
24	P	P	P	P	P	P	P	P	NA	P
25	P	P	NA	P	P	P	P	P	W	P
26	P	P	P	P	P	P	P	P	NA	P
27	P	P	P	P	P	P	P	P	NA	P
28	P	P	P	P	P	P	P	P	NA	W
29	P	P	P	P	P	P	P	P	P	P
30	P	P	P	P	P	P	P	P	P	P
31	P	P	NA	P	W	P	W	P	P	W
32	P	P	NA	P	P	P	P	P	W	W
33	P	P	-	P	P	P	P	P	P	P
34	P	P	P	P	P	W	P	P	P	P
35	P	P	P	P	W	P	P	P	P	P
36	P	P	P	P	P	P	P	P	P	P
37	-/+	-/+	P	W	P	P	P	NA	P	P
38	-	-	P	W	P	W	P	W	W	W
39	-	-	P	P	P	P	P	W	P	P
40	-	W	P	P	P	P	P	P	P	P
41	-	W	P	W	P	P	P	P	P	P
42	-	-	P	W	P	P	P	P	P	P
43	-	-	P	W	P	P	P	P	P	P
44	-	-	P	W	P	W	P	W	P	P
45	-	-	P	W	P	W	P	W	W	W
46	-/+	-	P	W	P	P	P	P	P	P
47	-	W	P	P	P	P	P	P	P	P
48	-	-	P	W	P	W	P	W	P	P
49	-/+	-	P	W	P	W	P	P	W	W
50	-/+	W	P	W	P	W	P	W	P	P
51	-	-	P	W	P	P	P	P	P	P
52	-	W	P	W	P	W	P	P	W	W
53	-	-	P	P	P	P	P	W	P	P
54	-	-	P	W	P	W	P	P	W	W
55	-	-	P	P	P	P	P	P	P	P
56	-	-	P	W	P	P	P	W	P	P
57	-	-	P	W	P	P	P	W	P	P
58	-	-	P	W	P	P	P	W	W	W
59	-/+	-/+	P	W	P	P	P	W	P	P

LM, laminin; Col, collagen; Dys, dystrophin; SG, sarcoglycan; DG, DG, negative (-); minimal (+/-), positive or normal (P) and decreased or irregular (W from weak); NA, not available.

Table 4. Statistical correlation between α-SG, γ-SG, β-DG, α-DG expression and presence or absence of merosin in patients' muscular biopsy.

Crosstabulation count α-SG, γ-SG β-DG,α-DG X merosin status				
		P	W	Total
α-SG	MD	5	18	23
p value 0,000	MP	27	5	32
	Total	32	23	55
γ-SG	MD	15	8	23
p value 0,099	MP	27	5	32
	Total	42	13	55
β-DG	MD	17	6	23
p value 0,766	MP	24	7	31
	Total	41	13	54
α-DG	MD	11	11	22
p value 0,003	MP	20	2	22
	Total	31	13	44

Significance: 0.05 (α=5%), SG, sarcoglycan; DG, dystroglycan; MD, merosin deficient; MP; merosin positive; P, positive; W, weak.

Table 5. Statistical correlation between α-SG, γ-SG β-DG expression and walking hability in MP patients.

Crosstabulation count: α-SG, γ-SG β-DG expression X walking hability					
		Walking with support	Independent walking	No walking	
α-SG	P	6	18	3	27
p value	W	0	3	2	5
0.183					
	Total	6	21	5	32
γ-SG	P	5	19	3	27
p value	W	1	2	2	5
0.249					
	Total	6	21	5	32
β-DG	P	6	17	1	24
p value	W	0	4	3	7
0.017					
	Total	6	21	4	31

Significance: 0,05 (α=5%); SG, sarcoglycan; DG, dystroglycan; MP, merosin positive.

Table 6. Statistical correlation between α-SG, γ-SG, β-DG expression and moderate endo-perimysial fibrosis in MP patients

Crosstabulation count: α-SG, γ-SG, β-DG expression X moderate endo-perimysial fibrosis									
		Moderate fibrosis							Total
		0	.5	1.0	1.5	2.0	3.0	4.0	
α-SG	P	0	3	3	18	1	1	1	27
p value	W	0	0	1	3	0	0	1	5
0.698									
	Total	0	3	4	21	1	1	2	32
γ-SG	P	0	3	4	17	1	1	1	27
p value	W	0	0	0	4	0	0	1	5
0.01									
	Total	0	3	4	21	1	1	2	32
β,-DG	P	0	3	3	18	0	0	0	24
p value	W	0	0	1	3	1	1	1	7
0.035									
	Total	0	3	4	21	1	1	1	31

Significance: 0,05 (α=5%), SG, sarcoglycan; DG, dystroglycan; MP, merosin positive; P, present; W, weak

Table 7. Statistical correlation between α-SG, γ-SG, β-DG expression and fatty infiltration in MP patients.

Crosstabulation count: α-SG, γ-SG, β-DG expression X fatty infiltration							
		Fatty infiltration					Total
		-	+	++	+++	++++	
α-SG	P	2	11	9	4	1	27
p value	W	0	3	1	1	0	5
0.874							
	Total	2	14	10	5	1	32
γ-SG	P	2	12	8	4	1	27
p value	W	0	2	2	1	0	5
0.939							
	Total	2	14	10	5	1	32
β-DG	P	2	10	10	2		24
p value	W	0	4	0	3		7
0.051							
Total		2	14	10	5		31

Significance: 0.05 (α=5%); SG, sarcoglycan; DG, dystroglycan; MP, merosin positive; - absent; + mild; ++ moderate; +++ marked; ++++ severe and widespread.

ty) (p = 0.017) and a marked fibrosis on muscle biopsy (p = 0.035) (Tables 5 and 6). The reduced expression of the α-SG, γ-SG and β-DG had no statistically significant correlation with fat infiltration (p = 0.874, p = 0.939 and p = 0.051, respectively) and fiber size variability (p = 0.375, p = 0.263 e p = 0.073, respectively) on muscle biopsy (Tables 7 and 8).

DISCUSSION

Regarding the MD patients, our aim was to evaluate the influence of the primary deficiency of merosin on the expression of the main proteins of the DGA complex, comparing their expression with that observed in MP patients. In the MP patients our intention was to select possible clinical particularities according to the protein expression.

Table 8. Statistical correlation between α-SG, γ-SG, β-DG expression and fiber size variability in MP patients.

Crosstabulation count: α-SG, γ-SG, β-DG expression X fiber size variability

		Fiber size variability				Total
		+	++	+++	++++	
α-SG	P	3	20	3	1	27
p value	W	0	3	2	0	5
0.375						
	Total	3	23	5	1	32
γ-SG	P	2	21	3	1	27
p value	W	1	2	2	0	5
0.263						
	Total	3	23	5	1	32
β-DG	P	3	19	2		24
p value	W	0	4	3		7
0.073						
	Total	3	23	5		31

Significance: 0.05 (α=5%); SG: sarcoglycan; DG, dystroglycan; MP, merosin positive; + mild; ++ moderate; +++ marked; ++++ severe and widespread.

Merosin - In 9 patients among 23 merosin deficiency was partial. All manifested the abnormal white matter on brain neuroimaging and clinical severity that characterize the MD patients with total absence of merosin[19-21]. Although patients with CMD due to mutations in other genes, particularly those related with abnormal glycosilation of α-dystroglycan can present a secondary partially deficient merosin[3,13,14], until the moment the association between widespread white matter changes and partial merosin deficiency has only been described in patients with mutations in the LAMA2 gene. In DMC1C (FKRPgene)[7], the partial deficiency of merosin is secondary to the marked deficiency of α-dystroglycan and is not associated with brain white matter changes. In the muscle-eye-brain forms of CMD, like Fukuyama and MEB, a secondary deficiency of merosin may occur; however, such forms are easily distinguished from classic MD-CMD with partial deficiency by their characteristics ocular and brain changes[1-3].

The adequate identification of the partial deficiency of merosin depends on the use of different antibodies that recognize different fragments of the protein[22,23]. The most useful antibodies are those reacting to the merosin fragments of 80 and 300 kDa[23]. In two of our 9 patients with partial deficiency, we defined that the deficiency was partial only after the utilization of the antibody against 300kDa, as with the antibody against 80 kDa, the merosin seemed totally absent. This result, indicating the better sensibility of the antibody against 300 kDa, is in agreement with other studies[24,25]. The possibility that not yet identified subtypes of CMD may also present secondary partial deficiency of merosin reinforces the need of a careful investigation of merosin status in the muscular biopsy of CMD patients.

Even presenting a total deficiency of merosin, two CMD patients (Cases 53 and 54) acquired independent walking, and the youngest of them still maintains it. This finding is not reported in the casuistics about CMD patients with total absence of merosin[20,26]. One of these three totally MD patients (Case 54) deserves a special comment, as her ability for walking independently, that had been lost at 4 years of age, was recovered and maintained for two more years under steroid therapy. In conclusion in our MD patients, the ability to walk independently was not fully related with the partial or total merosin deficiency and the degree of clinical severity was not related to any particular immunohistochemical finding.

Dystrophin – The expression of dystrophin in the patients' samples was normal in all, independently of the merosin status and of the degree of histopathological dystrophic changes. Therefore, the severe clinical and muscular involvement observed in CMD patients is apparently not due to an abnormal interaction between laminin and dystrophin. However, it must be emphasized that we evaluated the dystrophin expression by using antibodies against the C-terminal domain only and not against the N-terminal or the central region domains, that could interfere with the correct interpretation of the interaction between laminin and all the human dystrophin domains. In fact, Fardeau et al.[19], using antibodies against the central region and N-terminal dystrophin dominium, demonstrated that the primary merosin deficiency can induce a secondary dystrophin deficiency.

Sarcoglycan complex – The correct association among the α, β, γ, d and ε sarcoglycans within the sarcoglycan complex has been widely discussed and there is not a perfect agreement among different researchers[27-29]. More studies about the assembly and interactions of sarcoglycan/sarcospan complex will contribute to better clarify the muscle function. In our CMD patients, the normal β and δ-sarcoglycan expression agrees with the literature[30]. A statistically significant decrease of α-sarcoglycan expression was observed in our MD patients (Table 4), probably indicating a stronger interaction between that

protein and merosin, through their link with β-dystroglycan. In any case, the greater dystrophic changes seen in MD patients could also interfere with the a-sarcoglycan expression. We also found, a decrease of γ-sarcoglycan expression, mainly in the MD patients (34.7%), but without statistical significance (Table 4). This finding could also suggest a closer connection between γ-sarcoglycan and merosin; however, both MD and MP patients, who presented severe dystrophic pattern, also presented a great, but not clinically significant reduction of γ-sarcoglycan (Table 4). Therefore, future studies about the relation between sarcoglycan expression and dystrophic pattern would be necessary.

In relation to WW, the sarcoglycans and merosin expression is variable. In general there is a secondary deficiency in the expression of merosin and α-sarcoglycan[31]. In three patients we observed normal merosin and a reduction of β-SG in one and of γ-SG in another patient.

Dystroglycan complex – The dystroglycan gene codes for a proteic precursor that processed by a protease results in α e b-DG[32,33]. The β-DG has a trans membrane domain and is directly connected to the cystein portion of the dystrophin by the C-terminal[32]. The α and β-DG interact directly in the extracellular matrix.

Although a normal expression of β-DG in both MD and MP patients has been reported[34], as the β-DG interacts with the merosin through the α-DG connection, it could be expected that in the primary absence of the merosin, the β-DG could also be reduced. In fact, we observed such reduction in some patients, but the percent of MP and MD patients presenting reduction in the β-DG staining was almost the same (22.5% and 26%, respectively). Besides, in the MP patients the β-DG reduction was statistically correlated to the intensity of the dystrophic pattern (Table 6). This seems to suggest that the partial reduction of the β-DG in CMD patients is probably more related to the dystrophic pattern and other correlated factors than to the absence of merosin. Finally, the possibility that mutations in genes not yet defined encoding other proteins could also justify this secondary β-DG reduction should be stressed.

As demonstrated in other studies, the α-DG expression can be variable in MD patients[30]. In our cases, the α-DG was reduced in 50% of the MD patients and in only 10% of the MP patients. This difference was statistically significant (Table 4) and was dependent on the closer relation between merosin and α-DG, already emphasized by others[7,30,35].

Abnormalities in the α-DG glicosilation have been considered important in the pathogenesis of many forms of CMD: Fukuyama, MEB, WW, 1C and 1D[3,7,8,13, 30,35-37]. In most of these cases a secondary deficiency in merosin has been detected. We only found two MP patients with reduced expression of α-DG and their clinical phenotype did not correspond to the clinical description reported in the new CMD forms with α-DG glicosilation defects[7,8]. One of these patients has a marked involvement of the cervical musculature (Case 25) and the other (Case 32) presented focal changes in brain white matter and Type 1 diabetes mellitus. Both had normal expression of the merosin. A phenotype very similar to that observed in Case 32, except by the lack of Type 1 diabetes mellitus, was found in Case 31; however this patient had normal α-DG expression. It is important to emphasize that those cases with brain white matter changes, like our Cases 31 and 32, would need the study of the LAMA2 gene for ruling out any mutation that according to Tezak et al.[38] could lead to retention of large fragments of merosin. However, in the routine attendance the homogeneously clinical severity and the widespread brain white matter changes typical of CMD-1A, in addition to the low availability of molecular studies of the LAMA2 gene, lead to the diagnosis of MD-CMD.

The phenotype of our WW patients was markedly homogeneous and similar to that reported in the literature[39]. A neonatal severe involvement of muscles and CNS (type II lysencephaly) leading to death in the first two years of life was the rule. Recently mutations in the O-mannosyltransferase 1 have been identified[40], suggesting that the O-manose glicosilation would be implicated in the neuronal migration process at least in one part of the WW patients. Both α-DG and merosin expression, were found to be reduced in the CMD with glycosilation defects[7,35,41]; however we did not find the former abnormalities, perhaps because not all the WW cases are associated to O-mannosyltransferase gene (POMT1)[40].

Collagen VI – We tested the collagen VI expression in 7 MP patients with marked distal joints hyperlaxity and variable degrees of muscular involvement. In two, the collagen expression was absent and one of them was later diagnosed as Bethlem myopathy and taken off from the present series[18]. The other (Case 33) was the only patient in our ca-

suistics who was diagnosed as Ullrich CMD by molecular analysis[18]. The Ullrich phenotype, characterized by distal joints hyperlaxity associated with proximal joints contractures is clinically and genetically heterogeneous[42,43] and corresponds to Ullrich CMD, i.e. associated to mutations in one of the three collagen VI genes in about 40% of the patients[42].

Closing remarks – Unlike MD patients who present homogeneous clinical severity and neuroimaging changes, our MP patients had a variable motor impairment and in 6 of them we found CNS involvement, which we had in part already discussed in a previous work[44]. The CNS involvement was manifested by MR (Cases 6, 11, 12, 24), brain and cerebellar atrophy (Cases 4 and 12, respectively), as well as by focal and non specific changes of brain white matter (Cases 12, 31, 32). As has already been stressed, the finding of focal changes of the brain white matter in two MP patients (Cases 31, 32) not associated to other neurological abnormalities would suggest the need for molecular analysis of the LAMA2 gene for ruling out MD-CMD. However, the homogeneous and typical clinical/neuroimaging picture, as well as the use of two antibodies, against 80 and 300 kDa fragments of merosin, seems sufficient for defining the diagnosis of MD or MP-CMD. Two MP patients (Cases 6 and 24) had cataracts in addition to MR and this association had also been previously reported by us[45]. Concerning the MP patient with Type I diabetes mellitus (Case 32, already deceased) it is difficult to define whether that association was fortuitous or genetically determined. Therefore, we did not find in the MP patients who manifested the above mentioned uncommon findings of CNS and/or ocular involvement, as well as of type I diabetes mellitus, any specific change in the expression of any of the proteins from the DGA complex. Still within the MP-CMD subgroup, two patients had a rigid spine phenotype (Cases 14 and 23), and would need genetic confirmation to eventually be included in the subgroup of CMD with rigid spine linked to chromosome 1p[6]. Although the majority of our MP patients present a less severe phenotype and maintain the capacity to walk, two our patients (Cases 11 and 12) presented an intense motor and respiratory impairment, similar to those observed in the MD group. In these patients, the immunohistochemical analysis of the proteins from the DGA complex did not detect any abnormality.

In conclusion, although our study did not characterize any remarkable clinical-immunohistochemical correlation, we consider that an immunohistochemical analysis as complete as possible, should be performed, for establishing the differential diagnosis with other forms of children myopathies, while we await more accessible molecular methods. In addition, the analysis of the immunohistochemical expression of the proteins from muscle and extracellular matrix with a number of already available antibodies, is an easy procedure, that can contribute for a better understanding of the pathogenesis of the dystrophic muscle, as well as for selecting a particular molecular study.

Acknowledgements – We are grateful to Dr. Carsten G. Bonnemann for the molecular diagnosis of Case 33 to Dr. Stephan Kroger who kindly donated the antibody to α-DG.

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