# ANALYSIS OF MUSCLE CALPAIN-3 IN LGMD 2A

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Received: march 2008; Published: may 2008

**ABSTRACT.** Muscular dystrophies are a heterogeneous group of genetically determined disorders characterized by progressive muscle weakness and wasting with variable distribution and severity. The mode of inheritance, the age of onset, the involvement of particular skeletal muscle types, as well as variable serum concentration and the overall progression have been used to classify different forms of muscular dystrophy. Several proteins from sarcolemma (dystrophin, sarcoglycans, caveolin-3), and muscle fibres cytoplasm (calpain 3) have been identified. Most of these proteins appear to play a role in supporting the structure of muscle fibres and some of them are known to be involved in molecular signaling and biochemical processes. Mutations in theirs genes are responsible for different forms of muscular dystrophies. By our studies, the diagnosis of calpainopathy was obtained by identifying calpain-3 protein deficiency by western blotting. We report here an interesting case with LGMD 2A with calpain-3 absent and reduced gamma-sarcoglycan.

**Keywords:** limb girdle muscular dystrophy, dystrophin glycoprotein complex, genetics, calpain 3

## INTRODUCTION

Muscular dystrophies are a heterogeneous group of inherited muscular disorders that typically involve striated muscle, including both skeletal and cardiac tissue.

The identification of dystrophin with 20 years ago, the protein that is absent or markedly reduced in skeletal muscle of Duchenne muscular dystrophy (DMD) patients, was the first step taken towards clarification of the molecular pathogenesis of muscular dystrophies (Vainzof M. et al.,2003), and has led to the classification of these disorders in two groups: those muscular disorders involving dystrophin (dystrophinopathies) and those muscle disorders involving other known dystrophin associated proteins (LGMDs) or as of yet unidentified gene products (nondystrophinopathies) which account for a third to a fourth of DMD cases. (Hajdur & Kalia, 2000).

The limb girdle muscular dystrophies (LGMDs) are a heterogeneous group of progressive disorders mainly affecting the pelvic and shoulder girdle musculature, ranging from a severe form with onset in the first decade and rapid progression, to a mild form with later onset and slower progression (M Vainzof et al.,2003).

LGMDs are divided into seven autosomal dominant forms (LGMD 1A-G) and twelve autosomal recessive forms (LGMD 2A-M), forms with a lettering system denoting the chronology of locus identification (A to F for dominant and A to M for recessive LGMDs (Bushby & Beckmann, 1995). They are: calpain 3 for LGMD2A, (Richard I et al., 1995), dysferlin for LGMD2B, (Bashir R et al.,1998, Liu J et al., 1998), α-sarcoglycan (adhalin) for LGMD2D, (Roberds SL et al., 1994), β-sarcoglycan for LGMD2E, (Bonnemann CG et al., 1995, Lim LE et al., 1995), γ-sarcoglycan for LGMD2C, 16 Noguchi S et al., 1995), δ-sarcoglycan for LGMD2F, Nigro V et al.,1996 Nigro V et al., 1996, ) telethonin for LGMD2G, (Moreira ES et al., 2000), TRIM32 for LGMD2H, (Frosk P.et al.,2002), fukutin related protein for LGMD2I, (Brockington M et al., 2001) and titin for LGMD2J.

The autosomal recessive forms of LGMD (LGMD 2) are much more common, having a prevalence of 1:15 000, with geographical differences, (Nigro V. et al., 2003) represent 13-25% from total of LGMDs and the forms autosomal dominant 75 -90% (Bushby, 1999, Mathews and Moore, 2003) from total of LGMD-2, are generally milder relatively rare, representing less than 10% of all LGMD cases. (Hauser MA, et al. 2003) The involved proteins are very diverse and include sarcomeric, sarcolemmal and enzymatic proteins. These variations have their source in the diversity of primary deficiencies responsible for the disease. The emergence of an LGMD phenotype can result from mutations in any of – at least – 19 different genes. Only three (LGMD1A to C) out of the six LGMD1 causal genes are known so far (Messina, Speer, Pericak-Vance, &McNally, 1997; Starling,Kok, Passos-Bueno,Vainzof, & Zatz, 2004) whereas all but one of the causative genes have been identified for the 13 LGMD2 (Balci Brockington et al., 2001; Frosk et al., 2002; Hackman et al., 2002; Lim et al., 1995; Liu et al., 1998; Moreira et al.,2000; Nigro et al., 1996; Noguchi et al., 1995; Richard et al., 1995; Roberds et al., 1994). It is also important to keep in mind that mutations, even though sometimes strictly identical, of several LGMD deficient proteins can lead to very different

phenotypes (Bashir et al., 1998; Bonnemann et al., 1995).

*Limb-girdle muscular dystrophy type 2A* or calpainopathy is an autosomal recessive muscular disorders characterized by symmetric atrophy of the pelvic, scapular and trunk muscles, elevated serum creatin kinase (CK) and degeneration- regeneration pattern on muscle biopsy (Fardeau et al., 1996). It is the most common form of LGMD in European countries, where represents 40% of LGMD, and affects about 1: 100 000 inhabitants. It is caused by mutation in the CAPN3 gene, which comprises 24 exons, covers a genomic region of 50kb, is expressed as a 3.5 kb transcript, and is translated into 94-kDa protein with a proteolytic function (Richard I& all, 1995). Mutations in calpain 3 gene are responsible for LGMD 2A. The role of calpain- 3 in muscle and how the absence or deficiency of calpain 3 causes muscular dystrophy remain unknown.

Originally, it was described as a skeletal muscle-specific calpain isoform, and was proposed to play an important role in vivo, possibly by digesting and/or interacting with muscle-specific proteins (H. Sorimachi et al., 1993).

The diagnosis of LGMD increasingly relies on a combination of immunohistochemical and immunoblotting analyses, followed by DNA sequencing to identify the primary mutation, which is essential for the provision of accurate genetic and prognostic counseling.

The aim of our studies is to evaluate the expression of dystrophin and calpain by Western blotting. The study of calpain 3 protein in muscle at this time can only be carried out by Western blotting since the antibodies that are available have no immunoreactions on the sections.

## MATERIALS AND METHODS

### Human Muscle Samples from Normal and Disease Control Patients

Protein analysis in the diagnosis of muscular dystrophies is based on understanding the mutated proteins associated with different forms of muscular dystrophy.

Our muscle biopsies (gastrocnemius) were taken from patients affected by LGMD as a part of the routine diagnostic procedure under local anesthesia. The diagnosis of LGMD was based on clinical and histochemical findings. All patients had minimal to moderate limb weakness predominantly involving the proximal muscles, with elevated serum creatinekinase.

Muscle samples from normal control subjects were also obtained, with consent, from legs amputated at the knee (gastrocnemius). The biopsies samples were frozen in isopentan cooled in liquid nitrogen and stored at -80°C.

The two main methods used in our studies are: immunohistochemical and immunoblotting (western blotting). Both techniques use labeled antibodies to the specific muscle protein that is abnormally expressed in a particular muscular dystrophy.

### Immunohistochemical analyses

Seven micrometers thick tissue sections were incubated with primary antibodies diluted in BSA (bovine serum albumine) in PBS. After repeated washes with phosphate buffered saline (PBS), secondary antibody conjugated to peroxidase from SANTA CRUZ as applied. Following washes with PBS, the antibodies were visualized with DAB (diaminobenzidine) (Sigma). The samples were evaluated by light microscopy.

We used three mouse monoclonal antibodies against three domains of N-terminal-, rod-domain- and C-terminal- dystrophin (NCL-DYS1, NCL-DYS2, NCL-DYS3) and monoclonal antibody against utrophin (NCL-DRP 2), one to α-sarcoglycan (NCL-a-SARC), β-sarcoglycan (NCL-b-SARC), γ-sarcoglycan (NCL-g-SARC), δ-sarcoglycan (NCL-d-SARC) and merosin (NCL-MER) from Novocastra.

A total of 40 muscle biopsies from patients with clinical diagnosis of LGMD were tested for dystrophin, utrophin, sarcoglycans (α, β, γ) and merosin. Of these 40 patients, 8 these patients have a normal expression of above mentioned muscle proteins, and it was necessary to investigate calpain 3.

The study of calpain 3 protein in muscle at this time can only be carried out by Western blotting since the antibodies that are available have no immunoreactions on the sections.

### Polyacrilamide gel electrophoresis and Western blotting

The Western blot analyse that we use was modified in order to separate the large proteins, more then 200 kDa (egg. dystrophin) from the others under 150 kDa (egg. calpain 3). In this method the polyacrylamide gel system is a biphasic one. By this system, the large proteins are separated in the top part of the gel while and smaller proteins, in the bottom. After electrophoresis the gel was blotted, for 1 hour. We use a nitrocellulose membrane and Towbin buffer as a transfer buffer.

The monoclonal primary antibodies cocktail was applied on the same gel and the protein expression was visualized by using a chromogen method with Western Breeze. We used two mouse monoclonal antibodies against two domains of dystrophin: rod-domain (DYS-1) and C-terminal (NCL-DYS2), and one mouse monoclonal antibody for calpain 3 (NCL- CALP-2C4) (Novocastra).

With commercially available antibodies, calpain 3 is only detectable on blots and produces a characteristic pattern of bands.

For Western blotting, cryopreserved muscle tissues were homogenized in lysis buffer. Samples were electrophoretically separated on SDS-polyacrylamide gels. After electrophoresis, the proteins were transferred onto nitrocellulose membrane sheets. After blocking and washing, one of the membrane was incubated with a cocktail of monoclonal antibodies ones (NCL-CALP-2C4, NCL-DYS 2) and the other with (NCL-DYS-1 and γ- sarcoglycan) for an hour at room temperature. Follow that the membrane was incubated with peroxidase-conjugated anti-mouse


Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii (Life Sciences Series), vol. 18, 2008

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secondary antibody and the bands are visualized with DAB chromogen.

The amount of calpain-3 protein band (molecular weight 94 kDa) was normalized to the amount of tissue loaded, as determined by the skeletal myosin bands in the post-transfer Coomassie blue-stained gels (Coomassie stain of myosin heavy chain (MHC) protein demonstrates relative loading of muscle protein).(Nicholson LVB et al. 1990)

## RESULTS AND DISCUSSION

We identified 8 patients with limb-girdle muscular dystrophy 2A from 40 patients diagnosticated with progresive muscular dsytophy. One of these 8 patients with LGMD 2A is a special one, because calpain is absent on blot and reduced gamma-sarcoglycan. In a normal way, calpainopathy patients have normal dystrophin and sarcoglycan labelling on muscle biopsies.

Immunohistochemical analyses

At patients diagnosticated with LGMD 2A by immunohistochemical analysis, the proteins analysed have a normal expression. Utrophin (an autosomal protein that has considerable, sequence homology with dystrophin) was localized at the membrane of neuromuscular junction.

In one of the patients, a girl of 14 years old with clinical diagnosis of muscular dystrophy, the laboratory diagnosis was limb girdle muscular dystrophy 2C. After applying immunohistochemical technique we observed a very little labeling for gamma-sarcoglycan. But later, the Western blot analysis for Calpain3 demonstrated a total lack of this protein. In this case it was difficult to have a correct diagnosis: LGMD 2A or LGMD 2C and it was necessary to investigate calpain 3 by western blot analyses.

The other sarcolemmal proteins were normal expressed (immunohistochemistry).

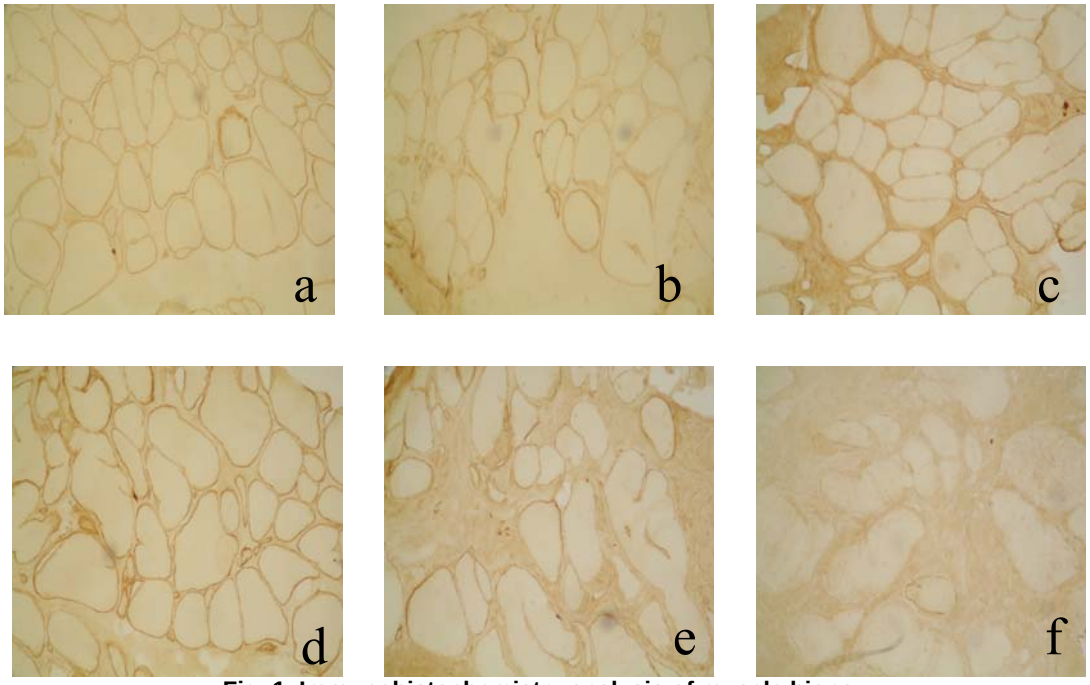


**Fig. 1 Immunohistochemistry analysis of muscle biopsy**

Normal immunostaining for Dys 1 (a), Dys2 (b) and Dys3 (c) (Ob. 10); Normal immunostaining for alfa-Sarcoglycan (d) and beta-Sarcoglycan (e) (Ob. 10); c) Innegal and faint immunostaining for gamma-Sarcogycan (f) (Ob. 10).

### Western blot analyses

On normal control subjects, when Dys C-terminal antibody was applied, a single band (427kDa) was detected, while for the rod domain of the protein we obtained a doublet (427 kDa and 400 kDa) plus lower molecular mass metabolites. At normal patients, *calpain 3* of the human sequence and recognized a single band at 94 kDa plus an additional clear band at about 30 kDa on blots.

For gamma sarcogycan the antibody recognized a single band at 35-kDa.

Fig 2. Shows a blot labelled for the *Dys1*, *Dys2* and *calpain 3* which was obtained after western blot analyses of four patients

We considered as normal bands whose intensity was the equivalent of control levels and abnormal those with a diminished intensity or bands that were absent.

- The patient in lane1 A and 1 B (Fig 2) - we have a normal expression of dystrophin and we observed that *calpain 3* is absent. *Gamma sarcoglycan* has a reduced expression on blot, too at 35 kDa. The patient was diagnosticated with LGMD 2A.
- The patient in the lanes 2A and 2B-has Limb girdle muscular dystrophy 2A. We can see the absence of *calpain 3* protein at this patient. Dystrophin is normal present on blot for both antibodies: Dys 1 and *Dys 2*.
- In the lane 3A and 3B –patient has Becker muscular dystrophy. We observed a complete absence of dystrophin labeling on blots for rod domain (*Dys 1*) and weakly signal (almost undetectable) for the antibody against to the

C-terminus rod dystrophin (*Dys 2*). This patient has a normal *calpain 3*.

- The patient in lane 4 A and 4 B we can see a complete absence of dystrophin labeling on blots for both antibodies (*Dys 1* and *Dys 2*) and a normal presence of calpain 3 on blot. The patient was identified with Duchenne muscular dystrophy.

The study of *calpain 3* protein in muscle at this time can only be carried out by Western blotting since the antibodies that are available have no immunoreactions on the sections. *Calpain 3* is stable in intact human muscle, there being little decrease in the abundance of full-size protein detected in biopsy samples rested at room temperature for up to 8 hours after leaving the body. This is in contrast to early reports that seemed to indicate rapid autolysis. (Shorimaki et al., 1996).

Western blot analyze detect bands at 94-kDa and an additional band at 30 kDa for patients with normal calpain 3. 8 Cases from 40 was diagnostic with LGMD 2A. At patients with limb-girdle muscular dystrophy 2A the bands of 94kDa and 30 kDa are absent. In the same time dystrophin has normal expression at these patients.

It is also necessary to test the patients with limb-girdle muscular dystrophy for dystrophin expression because is known that this protein is present at the patients with LGMD 2A.

Western blot analysis of muscle calpain 3 in LGMD 2A can show a total, partial, or more rarely, no apparent deficiently at all, with no direct correlation between the amount of calpain and severity of the phenotype.

LGMD 2 A patients with missense mutation may present a faint or almost normal 94-kDa calpain band (A, B) suggesting that some mutation may affect protein function without eliminating protein from muscle. A normal amount of calpain 3 on western blotting may be found in genetically proven calpainopathies, whereas calpain may be reduced in amount or absent in other forms of LGMD as secondary effect (Zatz, 2005).

This reduced expression of gamma-sarcoglycan is also seen by western analysis.

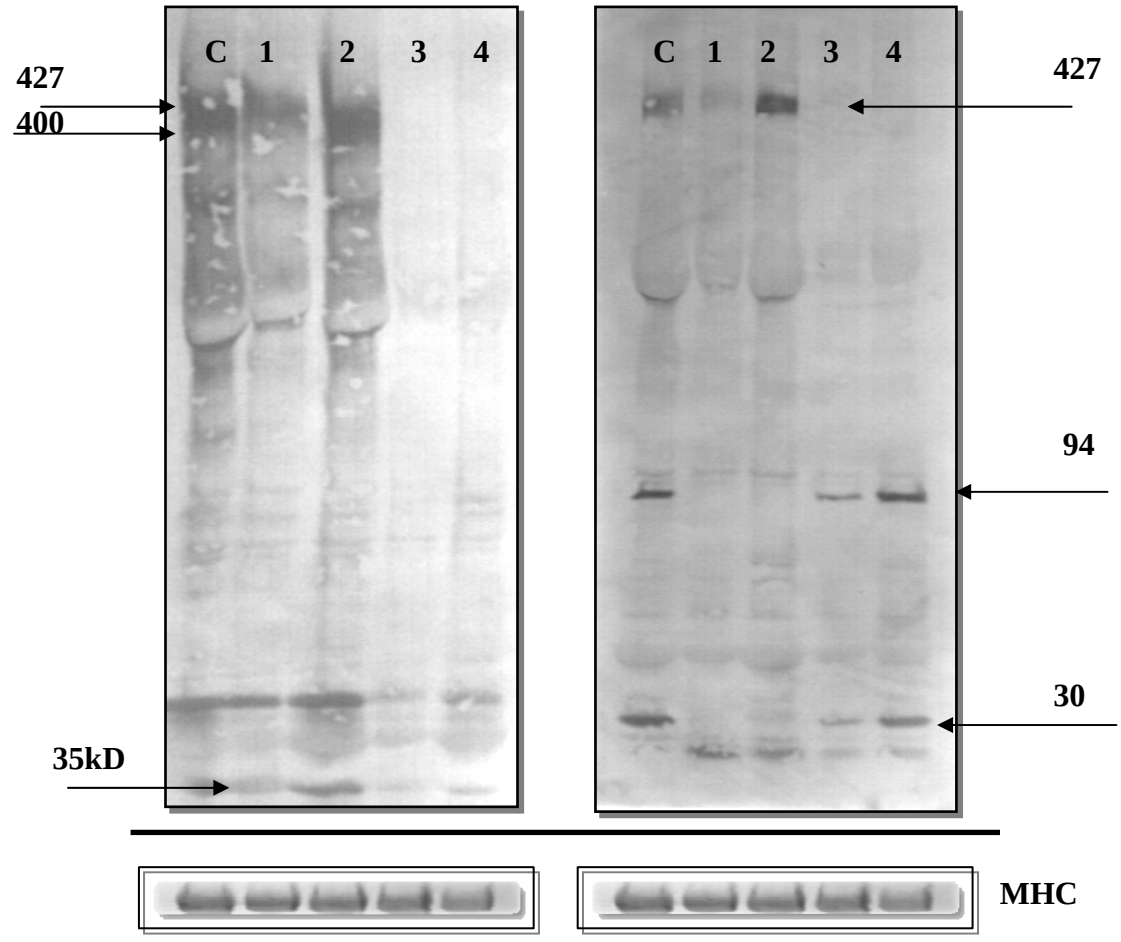


**Fig. 2. Western blotting analysis of muscle homogenates from four patients with muscular dystrophy**
**A.** Blot labelled with Dys1: C-normal control; lane 1-LGMD 2A, normal expression of dystrophin labelling, reduced expression of gamma-SG; lane 2-LGMD 2A; lane 3-DMD, absence of dystrophin labelling; lane 4-DMD, absence of dystrophin.
**B.** Blot labelled with Dys2 + calpain 3: C-normal control; lane 1-LGMD 2A, normal expression of dystrophin labelling, absence of calpain 3; lane 2-LGMD 2A; lane 3-DMD, absence of dystrophin labelling; lane 4-DMD, absence of dystrophin, reduced expression of calpain 3;
**MHC** – Corresponding myosin heavy chain bands on the post-blotted gel, stained with Coomassie blue

## CONCLUSIONS

In LGDM 2A immunohistochemical studies showed normal sarcolemmal labelling for dystrophin, utrophin and sarcoglycans.

We find a very interesting case with calpain 3 absent on blot and reduced gamma sarcogycan by immunohistochemical analyses. It is an unusual case because of the presence of two affected proteins that are not co- localised in the muscle cell. It is possible that the connection between the two proteins be made by filamin. It suggested a common pathological mechanism linking LGMD 2A with LGMD 2C-F (sarcoglycanopathies) and LGMD 1A caused by mutation in myotilin.

The study of calpain 3 protein in muscle at this time can only be carried out by Western blotting since the antibodies that are available have no immunoreactions on the sections.

Protein analysis using Western blotting with specific antibodies is obvious method for the differential diagnosis of muscular dystrophy and for elucidation of the physiopathology of each genetic disorder involved.

A large proportion of cases can be resolved by detailed clinical assessment followed by combined analysis of muscle proteins by both immunoblotting and immunohistochemistry. Using a full range of diagnostic antibodies undoubtedly improves the diagnostic of muscular dystrophy.

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