Disease progression in myotonic dystrophy type 2: histopathological and molecular parameters from muscle biopsies

M. Giagnacovo,[1] R. Cardani R,[2,3] G. Meola[2,4]

[1]Dipartimento di Biologia e Biotecnologie "Lazzaro Spallanzani", Laboratorio di Biologia cellulare e Neurobiologia, Università di Pavia; [2]Laboratorio di Istopatologia Muscolare e Biologia Molecolare, IRCCS Policlinico San Donato, Università degli Studi di Milano; [3]Fondazione Malattie Miotoniche-FMM Milano; [4]Dipartimento di Neurologia, IRCCS Policlinico San Donato, Università degli Studi di Milano, Italy

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Corresponding author: Marzia Giagnacovo
Dipartimento di Biologia e Biotecnologie, Università degli Studi di Pavia, via A. Ferrata 9, 27100 Pavia, Italy.
Tel. +39.0382.986417 - Fax +39.0382.986325.
E-mail: marzia.giagnacovo@unipv.it

Summary
Myotonic dystrophy type 2 (DM2) is a dominantly inherited autosomal disease with multisystemic clinical features and is caused by expansion of a CCTG tetranucleotide repeat in the first intron of the zinc finger protein 9 (*ZNF9*) gene in 3q21. The expanded-CCUG-containing transcripts are retained in cell nuclei, as ribonuclear inclusions, which specifically sequester some splicing factors (as MBNL1), thus causing a general alteration of the pre-mRNA post-transcriptional pathway that is likely responsible for the DM2 multifactorial phenotype. DM2 is a disease of type 2 fibers and it is has been hypothesized that the symptom worsening that occurs with aging may be caused by the progressive accumulation of CCUG-expansion and the sequestration of protein factors. Thus, we carried out a morphometric analysis of ribonuclear inclusions and MBNL1 *foci* in muscle sections from three DM2 patients who underwent two successive biopsies, and we studied the evolution of the histopathological features. Increase in size and fluorescence intensity has been observed in MBNL1 *foci*, together with a worsening of muscle histopathological traits, such as type 2 fibers impairment.

Key words: myotonic dystrophy type 2-DM2, ribonuclear inclusions, MBNL1 *foci*, fluorescence microscopy, confocal microscopy.

Introduction

Myotonic dystrophies (DM) are genetically-based neuromuscular disorder characterized by muscle hyperexcitability (myotonia) and a wide spectrum of multisystemic traits (Meola *et al.*, 1999; Moxley *et al.*, 2002; Meola, 2000; Day *et al.*, 2003; Udd *et al.*, 2003), among which muscular dystrophy with increased number of clumped or centrally located nuclei in skeletal muscle fibers (Vihola *et al.*, 2003).

Two DM forms exist, i.e. the more severe DM1-Steinert's disease (OMIM 160900) and DM2 (OMIM 602688).

DM1 depends on the expansion of a (CTG)n nucleotide sequence in the 3' untranslated region of the Dystrophia Myotonic Protein Kinase (*DMPK*) gene (Brook *et al.*, 1992; Fu *et al.*, 1992; Mahadevan *et al.*, 1992).

DM2 exhibits a milder clinical phenotype and depends on the expansion of the tetranucleotidic repeat (CCTG)n in the first intron of the Zinc Finger Protein (*ZNF*)-9 gene (OMIM 116955) (Liquori *et al.*, 2001) on chromosome 3q21 (Ranum *et al.*, 1998). The expanded-CCUG containing transcripts are retained in the nucleus, in the form of mutant RNA *foci*, also called ribonuclear inclusions (Liquori *et al.*, 2001; Mankodi *et al.*, 2001, 2003), where essential splicing factors such as muscleblind-like (MBNL) proteins (Fardaei *et al.*, 2002; Cardani *et al.*, 2006; Pascual *et al.*, 2006), snRNPs and hnRNPs (Perdoni *et al.*, 2009 a,b) are sequestered. As a consequence of the nuclear depletion and loss of function of these regulators (Mankodi *et al.*, 2001), a general alteration of the pre-mRNA post-transcriptional pathway takes place, which could account for the multifactorial phenotype of DM2 patients (Wheeler and

Thornton, 2007; Ranum and Cooper, 2006).

DM2 has been defined as "a disease of type 2 fibers" since these myofibers are selectively affected by atrophy and central nucleation (Bassez *et al.*, 2008, Vihola *et al.*, 2003); consistently, muscles which mostly contain type 2 fibers (such as proximal skeletal muscles) are the most compromised ones. The presence of centrally located nuclei and pyknotic nuclear clumps (clusters of heterochromatic myonuclei considered the end product of atrophy due to denervation) are peculiar histopathological features of this disease (Schoser *et al.*, 2004).

For diagnosing DM2, it is crucial to detect the characteristic histopathological traits of type 2 muscle fibers (Meola *et al.*, 2011), although the most rapid, sensitive and decisive technique is the identification of the expanded RNAs in the nuclear *foci* by fluorescence in situ hybridization (FISH) or the immunolabeling of MBNL1 sequestered in the ribonuclear inclusions (Cardani *et al.*, 2004, 2006).

In DM2 (and more generally in DMs), the symptoms undergo worsening in aging patients, and it has been hypothesized that this may be caused, at the cellular level, by the progressive accumulation in the nucleus of expanded RNAs and the sequestration of protein factors involved in RNA processing and export to the cytoplasm: this would likely result in the presence of progressively larger intranuclear *foci* with increasing age.

To test this hypothesis, we performed a morphometric analysis of the nuclear *foci* in muscle sections of *biceps brachii* from three DM2 patients who underwent two successive biopsies; in parallel, the evolution of the muscle histopathological features was also considered. We chose this muscle because it is composed mostly of type 2 fibers, which are mainly compromised in DM2 disease.

Materials and Methods

Patients

Samples of *biceps brachii* were taken under sterile conditions from three clinically diagnosed DM2 patients, after informed consent, at two different times (age at the first biopsy: 54, 37, and 33; age at the second biopsy: 58, 47, and 38, respectively). The protocols were approved by the ethical committee of the IRCCS Policlinico San Donato, Milan, Italy. The muscle samples were frozen in isopentane and stored in liquid nitrogen until use. Serial cryostatic sections were used for all the procedures reported below.

Evaluation of some histopathological features in DM2 muscle sections and immunolabeling of fast, type 2 muscle fibers

To evaluate basic tissue and cell organization (fiber atrophy, morphological and structural alterations of muscle cells), some cryostatic sections were either conventionally stained with hematoxylin and eosin or processed for ATPase pH 10.0 staining or immunolabeled for the fast myosin heavy chain, to detect type 2 fibers, as follows.

Since muscle are composed by various fiber types, a myosin adenosine triphosphatase (ATPase) stain at pH 10.0 was performed to label type 2 fibers (Round *et al.*, 1980), mostly compromised in DM2. Cryostat sections (6 µm) were incubated with ATP incubation solution, pH 10.0, at 37°C for 45 min, then samples were washed in 1% calcium chloride solution, in 2% cobalt chloride solution, and in distilled water. Then, sections were incubated for 30 sec in 1% ammonium sulfide solution and washed in distilled and tap water. Nuclei were counterstained with haematoxylin, the samples were dehydrated in ethanol-xilene and finally mounted in Eukitt (Sigma-Aldrich, Buchs, Switzerland).

To immunolabel fast-myosin, six-µm-thick cryostat sections were air-dried at room temperature (RT), rehydrated in phosphate buffere-saline (PBS) and incubated for 20 min at RT with normal goat serum (NGS; DAKO, Glostrup, Denmark) diluted 1:20 in PBS containing 2% bovine serum albumin (BSA; Sigma-Aldrich) to block aspecific binding sites. The sections were then incubated for 1 hour at RT with a mouse monoclonal antibody recognizing fast myosin heavy chain isotype (Sigma-Aldrich) diluted 1:400 in PBS containing 2% BSA, washed in PBS and incubated with a goat anti-mouse biotinylated secondary antibody (Sigma-Aldrich) (1 hour incubation at RT, dilution 1:300 in PBS); the slides were finally incubated with peroxidase-conjugated streptavidin (Vectastain Elite ABC kit, Burlingame, Canada) revealed with 3-3' diaminobenzidine tetrahydrochloride (DAB; Sigma-Aldrich), counterstained with Mayer's haematoxylin (to visualize nuclei), dehydrated in ethanol-xilene and mounted in Eukitt (Sigma-Aldrich).

Control sections were processed as above, but

omitting incubation with the primary antibody.
Nuclear clumps and centralized nuclei were counted, by using the program ImageJ (Scion Corporation, USA), in cross-sections immunolabeled for fast myosin and the results were expressed as the number of nuclear clumps or centralized nuclei/mm^2. The relative atrophy and hypertrophy factors (increasing values indicate abnormal myofiber size distribution) were also estimated: the minor axis of 100 myofibers per sample were measured and, by using the program Microcal Origin (Microcal Software Inc., Northampton, MA), the histogram of axis length distribution and the curve according to Gauss equation were obtained. Then, according to Dubowitz *et al.*, (1985), the Gauss curves of our samples were compared with the standard distribution of myofiber size in male and female healthy populations.

Dual-labeling of nuclear *foci* by fluorescence in situ hybridization (FISH) and immunofluorescence

The FISH procedure was carried out on muscle sections as previously reported by Cardani *et al.*, (2004). In brief, 6 µm thick transverse cryostatic sections were air dried for 30 min and fixed with 2% paraformaldehyde for 30 min at 4°C. The sections were then washed in PBS and permeabilized in 2% acetone in PBS, pre-chilled at -20°C, for 5 min. After washing in PBS, sections were incubated in 40% formamide and 2x saline solution citrate (SSC) for 10 min at RT, and hybridized for 2 h at 37°C, with 1 ng/µL probe $(CAGG)_5$ Texas red labeled probes (IDT, Coralville, IA) in 30% formamide, 2xSSC, 0.02% BSA, 67 ng/µL yeast tRNA, 2 mM vanadyl ribonuclease complex (all these reagents were from Sigma-Aldrich). The sections were washed first in 40% formamide and 2x SSC at 45°C for 30 min, then in 1x SSC at 45°C for 15 min and another 1x SSC wash at RT.
The FISH-labeled sections were then processed for the immunofluorescence detection of MBNL1, as follows. The sections were pre-incubated for 20 min at RT with 5% NGS (Dako) in PBS containing 2% BSA (Sigma-Aldrich), and then incubated overnight at 4°C with a rabbit polyclonal antibody recognizing MBNL1 (kind gift of Prof. C.A. Thornton, University of Rochester, New York, USA); the primary antibody was diluted 1:1000 in PBS containing 2% BSA. The sections were washed in PBS and then incubated for 1 h at RT with a goat anti-rabbit Alexa488-labeled antibody (Molecular Probes; Invitrogen, Milan, Italy), diluted 1:200 in PBS; counterstained for DNA with Hoechst 33258 (1 µg/mL; Sigma-Aldrich), and mounted with Mowiol (Calbiochem, Milan, Italy). As controls, some slides were processed as described above but omitting the incubation with the primary antibody.

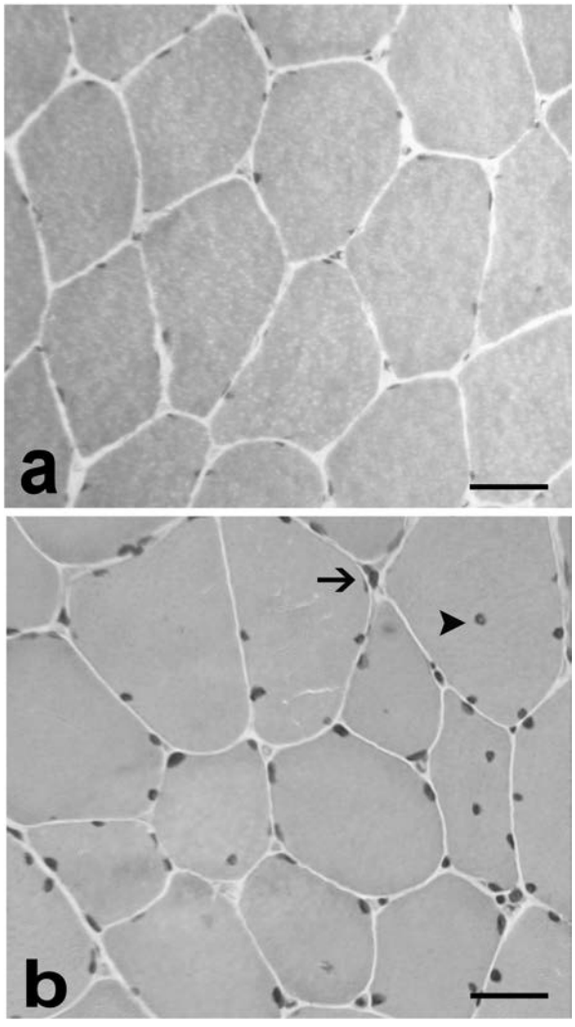

Figure 1. Haematoxylin and eosin staining of *biceps brachii* sections from healthy (a) and DM2 patients (b). In DM2 patients fiber size variation, centrally located nuclei (arrowhead) and pyknotic nuclear clumps (arrow) are apparent. Bars: 50 µm.

Morphometric evaluation of the ribonuclear inclusions and MBNL1-containing *foci*

Dual-labeled sections by FISH and MBNL1 were analyzed by confocal microscopy, using a Leica TCS SP2 AOBS system: for fluorescence excitation, an Ar/Vis laser at 488 nm for Alexa488, and He/Ne laser at 543 for Texas red; the laser intensity, pinhole opening, signal amplification and image spacing along the z axis were kept constant, and images were recorded in the 1024×1024 pixels format, using a 63x oil immersion objective. For each section, 60 ribonuclear inclusions and the corresponding MBNL1-containing *foci* were measured. Acquisitions were carried out at the Centro Interdipartimentale di Microscopia Avanzata (CIMA) of the University of Milan, Italy. The size and fluorescence intensity of the ribonuclear inclusions and MBNL1 *foci* were measured using the Leica Confocal Software. The mean values and standard errors were calculated; statistical analysis was made through ANOVA test.

Results

In Figure 1, examples of *biceps brachii* sections from a healthy and DM2 patient after hematoxylin and eosin staining are reported: apparently, in DM2 muscle the fiber size is more variable than in muscle from a healthy subject, and centrally located nuclei and pyknotic nuclear clumps are present. These histopathological features mostly occur in type 2 fibers (Figure 2a, b), and become

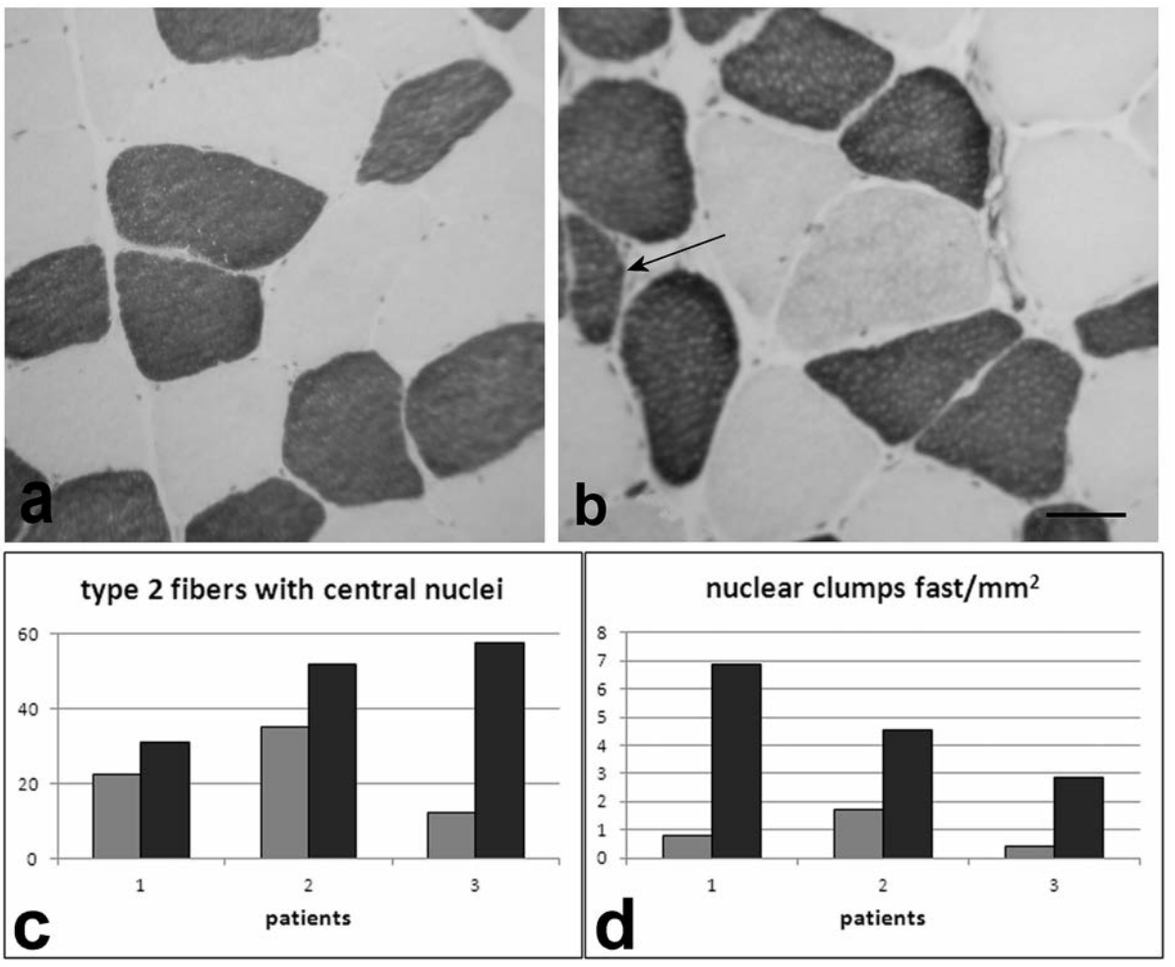

Figure 2. ATPase pH 10.0 reaction for type 2 fibers on *biceps brachii* sections from a healthy (a) and a DM2 (b) patient: fiber size variation and atrophic fibers (arrow) are shown. Bars: 50 µm. (c, d) Histograms showing the number of type 2 fibers with centralized nuclei and the number of nuclear clumps/mm^2 in fast myosin-positive fibers. Gray columns refer to the first biopsy, black ones to the second. In all patient the two parameters increased from the first to the second biopsy.

more frequent with increasing patient's age, as shown in Figure 2 (c, d), where these characteristics were quantitatively compared between the first and second biopsy of each patient. The relative atrophy and hypertrophy factors in type 2 fibers, as estimated by morphometric analysis, proved to increase in the second biopsy of all patients, with the only exception of the hypertrophy factor in patient 1 (Table 1).

Figure 3 shows the co-localization, in the nuclear *foci* of DM2 patients, of the FISH signal (Figure 3b) for expanded RNA and the immunopositivity for MBNL1 (Figure 3c). The areas of FISH or MBNL1 positivity, and the corresponding fluorescence intensities were measured in confocal images, considering muscle sections obtained from the two successive biopsies of each patient. In all patients, a significant increase was observed for both ribonuclear inclusions and MBNL1 *foci* in the second biopsy compared to the first one. As shown in Figure 4, the areas of FISH positive *foci* increased by about 37%, 29% and 20% in the second biopsy of patient no. 1, 2 and 3 respectively, while the areas of MBNL1 *foci* in the second biopsy were respectively larger by about 73%, 23% and 38%. Similar trends were found for the corresponding fluorescence intensities of FISH and MBNL1, which increased in the second biopsy by about 17% and 69% in patient 1, by about 16% and 20% in patient 2, and by about 22% and 38% in patient 3.

Table 1. Relative atrophy and hypertrophy factors in type 2 fibers of *biceps brachii* from the three patients considered in our study. Both factors increase in the second biopsy in all patients, with the only exception of the hypertrophy factor in patient 1.

	Relative atrophy factor		Relative hypertrophy factor	
	First biopsy	Second biopsy	First biopsy	Second biopsy
Patient 1	1.00	1.17	3.05	0.55
Patient 2	0.20	1.63	4.81	6.13
Patient 3	0.29	1.12	0.72	2.52

Discussion and Conclusions

The expansion of the CCTG repeat in first intron of *ZNF9* gene has been associated to the multisystemic pathological features of DM2 patients

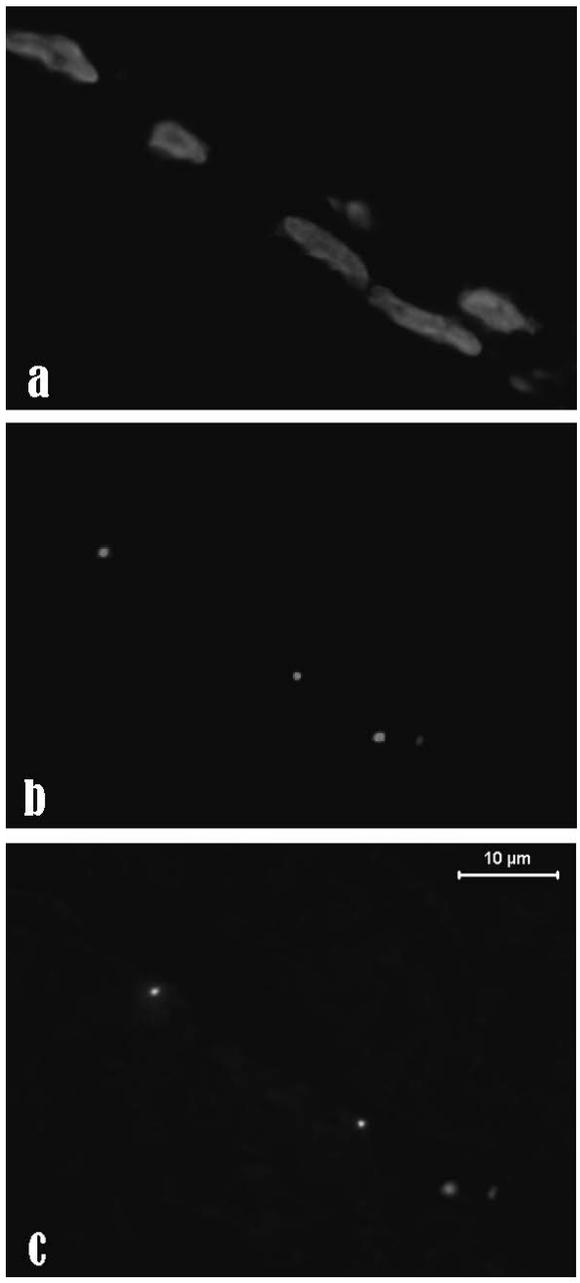

Figure 3. FISH for expanded RNA and immunofluorescence for MBNL1 on DM2 *biceps brachii* sections. (a) DNA was counterstained with Hoechst 33258; (b) ribonuclear inclusions and (c) MBNL1 *foci* colocalize. Bar: 10 µm.

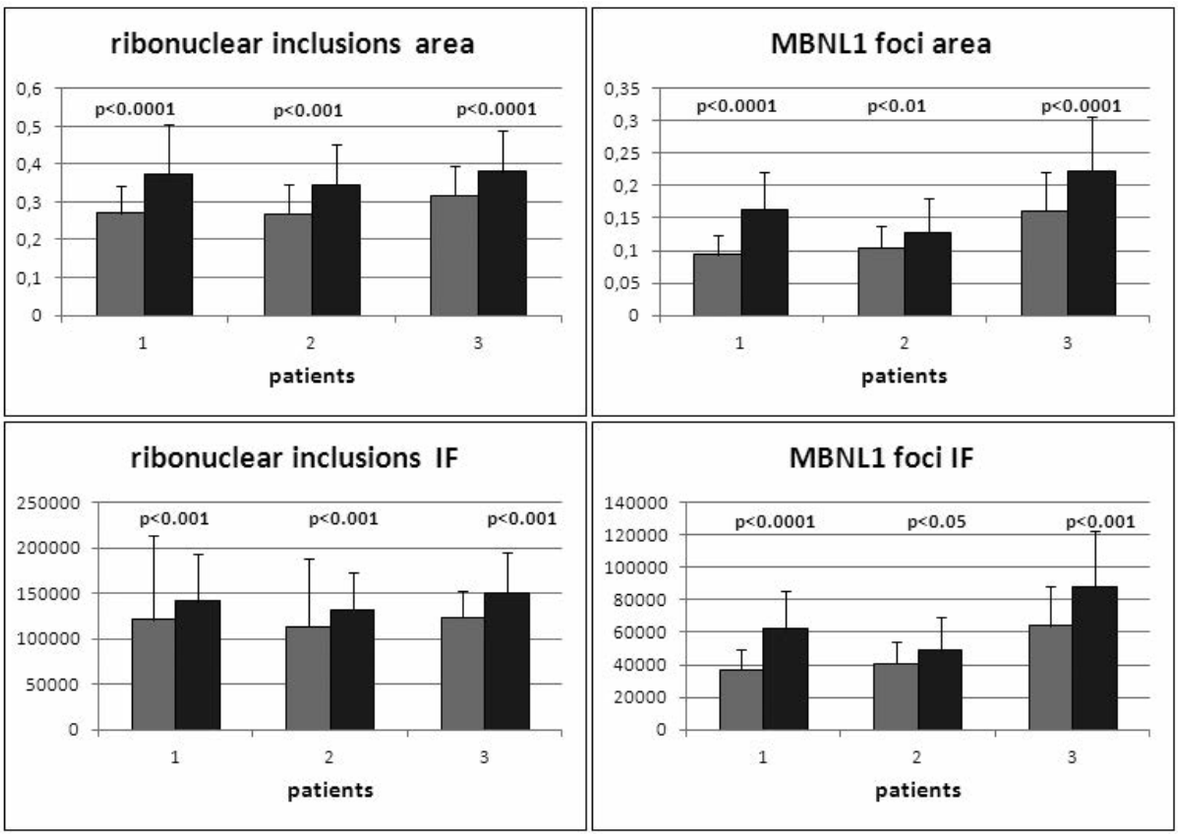

Figure 4. Quantitative mean ± standard errors of dimensions and fluorescence intensity (IF) of ribonuclear inclusions and MBNL1 *foci*. Histograms showing a statistically significant increase of dimensions and fluorescence intensity from first to second biopsy in all patients considered. Gray columns refer to the first biopsy, black ones to the second. In all patient the two parameters increased from the first to the second biopsy.

(Ranum *et al.*, 1998; Liquori *et al.*, 2001). The expanded-CCUG containing transcripts are retained and accumulate in the nucleus, in the form of ribonuclear inclusions (Liquori *et al.*, 2001; Mankodi *et al.*, 2001, 2003) where several splicing factors essential for mRNA processing are sequestered (Fardaei *et al.*, 2002; Cardani *et al.*, 2006; Pascual *et al.*, 2006; Perdoni *et al.*, 2009).

It has been observed that clinical symptoms such as proximal muscle weakness and myotonia undergo progressive worsening with increasing age in DM2 as well as in DM1 patients (Meola *et al.*, 2002), and that the expansion of DNA repeats increased by approximately 2 kb in the leukophocytes of a DM patient during the 3-year interval between two successive blood donations (Liquori *et al.*, 2001).

Our results demonstrate that, in the nuclei of skeletal muscle cells from *biceps brachii* of three different DM2 patients who underwent two successive biopsies (at intervals of 4 to 10 years), the intranuclear *foci* increased in size and in content of expanded RNA and MBNL1, as estimated from the area and fluorescence intensities of the final reaction products after FISH or immunolabeling procedures.

We have recently demonstrated, using primary fibroblast cultures from DM2 patients as a model system, that upon exit from the cell cycle fibroblasts accumulate increasing amounts of MBNL1 in their nuclei, with a concomitant increase in size of the RNP-containing *foci*; we have hypothesized that in non-dividing cells the continuous and progressive sequestration of factors needed for RNA processing would lead to the onset and the worsening with time of the cell pathological traits in DM2 patients. The results of the morphometric analyses performed on the skeletal muscle biopsies from the three DM2 patients of the present investigation are compatible with this view: in

adult skeletal muscle, non-renewing cells are mainly present in which a progressive sequestration of protein factors needed for the mRNA processing apparently takes place with increasing patient's age. In parallel, there is a progressive worsening of the histopathological traits, such as pyknotic nuclear clumps and centrally located nuclei, and an increase in the atrophy/hypertrophy factors in type 2 muscle fibers which are known to be especially affected in DM2.
The close relationship between the amount of the RNP-containing *foci* and the extent of the pathological cell phenotype has been confirmed by experiments *in vitro* (Warf *et al.*, 2009) and in vivo (Mulders *et al.*, 2009), aimed at decreasing the intranuclear accumulation of MBNL1: this resulted in the reduction in the number and size of nuclear *foci*, and the restoration of the normal splicing processes, with attenuation of the diseased phenotype of dystrophic (DM1) cells.
As a final remark, it is worth recalling that in DM patients not only skeletal muscle is affected, but also the central nervous system and heart, where non-renewing cells are mainly present.

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