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MYOTONIC DYSTROPHY: A NEW PERSPECTIVE ON THE TREATMENT OF A MULTISYSTEMIC DISEASE

C. Marra[1], D. Quaranta[1], G. Silvestri[1,2] and A. Modoni[1]

[1]Department of Neuroscience, Catholic University of Sacred Heart, Rome, Italy; [2]Fondazione Don Carlo Gnocchi, Rome, Italy

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SUMMARY

So far, myotonic dystrophy (DM) has been linked to two distinct loci: DM1, associated with an abnormal CTG triplet expansion in the 3'-untranslated region of the myotonin-protein kinase gene (DMPK), and DM2, associated with an expanded CCTG repeat in intron 1 of the gene encoding zinc finger protein 9 (ZNF9). Both forms of DM are characterized by multisystemic involvement, affecting the skeletal muscle, heart, eyes, endocrine system and central nervous system (CNS). Currently, there is no effective therapy for DM; treatment is limited to supportive care to alleviate symptoms and avoid cardiac arrhythmic disorders. Moreover, data on DM therapies are quite empirical and very few randomized studies have been planned in the past years. Nevertheless, research has greatly improved our knowledge about biochemical pathways involved in the pathogenesis of this multisystemic disorder, giving rise to new therapeutic approaches acting on different molecular pathways involved in DM at the DNA, RNA or protein level. In this review, we summarize the clinical aspects of DM1 and DM2, with particular attention to the CNS involvement in DM1. We also briefly review the pathogenesis of these diseases, and finally, attempt to summarize some new perspectives on their treatment.

CLINICAL ASPECTS OF MYOTONIC DYSTROPHY TYPE 1

Myotonic dystrophy type 1 (DM1; #OMIM 160900) also known as Steinert's disease, is the most common muscular dystrophy, with a prevalence of 1:8,000. The disease is associated with an abnormal CTG triplet expansion at the 3'-untranslated region of the myotonin-protein kinase gene (*DMPK*) on chromosome 19q13.3, ranging from 50 to 4,000 CTG triplets (1).

Clinically, DM1 is a multisystemic disorder that affects the skeletal muscle, the heart, the eyes, the endocrine system, smooth muscle and the central nervous system (CNS) (2). Usually, the onset of symptoms occurs during adulthood or infancy, but congenital forms have also been described, especially in the case of maternal inheritance.

Muscle symptoms are represented by myotonia, weakness and atrophy predominantly affecting cranial (in particular facial, jaw and pharyngeal compartments), axial and distal limb muscles. Cardiac involvement is particularly relevant and consists mainly of a degeneration of the conduction tissue that may lead to the occurrence of life-threatening arrhythmias (3). Eye involvement is characterized by the development of premature cataracts (4). Glucose intolerance, thyroid dysfunction and fertility problems, in particular testicular atrophy, represent the most common endocrine disorders described in DM1 patients (2). Serological abnormalities such as γ-glutamyltransferase and creatine kinase elevations, and reduced immunoglobulin G (IgG) and IgM levels are also common (5).

Involvement of the bowel smooth muscle causes the occurrence of gastrointestinal symptoms, such as dysphagia, dyspepsia, constipation or diarrhea and incontinence, disturbances that are variably reported by up to 70% of patients (6). DM1 patients also display a wide spectrum of sleep disorders, mainly daytime sleepiness and fatigue, the most common nonmuscular symptoms (experienced by 80% of patients) (7).

CNS involvement in DM1

The clinical spectrum of CNS involvement in DM1 may range from a condition of mental retardation observed in patients affected by the congenital form of the disease (2), to behavioral aspects and cognitive changes, especially involving abstraction and visuospatial abilities in classic adult-onset forms of the disorder (8).

In particular, the occurrence of two different patterns of cognitive impairment in congenital vs. adult DM1 patients has been highlighted (9). In this regard, we may hypothesize that the early presence in brain tissues of congenital cases, either of alleles containing very

Correspondence: C. Marra, Department of Neuroscience, Catholic University of Sacred Heart, Rome, Italy. E-mail: cmarra@rm.unicatt.it.

long CTG repeats or of their corresponding RNAs, could alter the expression of genes regulating later stages of brain development, leading to a condition of mental retardation. Conversely, the increased levels of *DMPK* RNAs with very long CUG repeats, caused by a progressive abnormal CTG triplet expansion in brain tissue of adult-onset DM1 patients, could specifically affect the correct splicing of different genes in neurons of the frontal and temporal cortex, causing a progressive deterioration of frontal and executive functions with time (10). Involvement of frontotemporal structures was recently confirmed by Takeda et al. (11), who demonstrated a reduced sensitivity to facial emotions in DM1 subjects, concomitant with lesions in the anterior temporal white matter, the amygdala, and the insular and orbitofrontal cortices.

As a matter of fact, several lines of evidence support a pathological CNS involvement in DM1 similar to in other dementias. Eosinophilic intracytoplasmic ubiquitin inclusions have been observed in thalamic nuclei and the substantia nigra (12). Such inclusions are also composed of microtubule protein fragments and this association has been found in some other neurodegenerative disorders, such as corticobasal degeneration and dementia associated with amyotrophic lateral sclerosis. Moreover, neurofibrillary tangles comparable to those observed in Alzheimer's disease have been demonstrated in DM1 affecting the hippocampus, temporal lobes and entorhinal cortex. However, microtubule-associated protein tau precipitates are not linked to amyloid deposits in this case, as opposed to in Alzheimer's disease (13).

The prevailing deposition of tau and ubiquitinated proteins and the prevalence of deposits in temporal, frontal and limbic areas are in accordance with the observation of a prevalent impairment of executive and linguistic functions in adult forms of DM1 over time (10), suggesting the occurrence of a cognitive decline similar to that observed in frontotemporal dementia.

In particular, exons 2, 6 and 10 of tau proteins have been demonstrated to be misspliced in brain tissue from DM1 patients; the altered expression pattern of the tau isoform probably affects microtubule stabilization in the membrane region of neuronal cells, impairing their function (14). Such data support the hypothesis that the dementia during DM1 could be considered as a particular form of tauopathy in a recent classification of frontotemporal dementia (15).

Moreover, neuroradiological studies show nonspecific pathological findings such as ventricular enlargement, lobar and periventricular white matter lesions or cerebral atrophy (16). More recently, diffusion tensor imaging has been applied to DM1, documenting an involvement of the genu, rostral body, anterior midbody, posterior midbody and splenium of the corpus callosum, associated with a reduced volume of cortical regions connected by these fibers (17).

CLINICAL ASPECTS OF MYOTONIC DYSTROPHY TYPE 2

A second form of myotonic dystrophy, myotonic dystrophy type 2 (DM2; #OMIM 602668) has been associated with an abnormal CCTG expansion in intron 1 of the gene encoding zinc finger protein 9 (*ZNF9*) on chromosome 3q (18).

Although DM2 patients usually have longer $(CCTG)_n$ expansions (n ranging from 75 to 110,000) when compared with DM1 patients, the disease course seems to be milder than DM1, and up to now, no congenital forms have been described in the literature.

Indeed, DM2 shares the core features of DM1, including autosomal dominant inheritance, progressive muscle weakness, myotonia and multisystem involvement, with cardiac involvement, iridescent cataracts, male hypogonadism, insulin insensitivity and hypogammaglobulinemia (19).

Despite these striking similarities between DM2 and DM1 as multisystemic disorders, there are important differences as well. In particular, in patients with DM2, muscle weakness is more pronounced in proximal muscles, especially in lower limbs and axial muscles. Moreover, DM2 patients often complain of proximal muscle pain and tenderness. Furthermore, myotonia is less severe in DM2 than in DM1 and is often detected only at neurophysiological examinations, with a different distribution in DM2 patients than in individuals affected with DM1 (20).

In addition, the multisystem involvement seems to be much less severe in DM2 patients than in subjects affected with DM1. Indeed, cardiac conduction abnormalities are more rare in DM2 than in DM1 (21), while a progressive dilatative cardiomyopathy in the absence of overt myocardial ischemia or other obvious causes, has been described in DM2 as a potentially life-threatening condition (22).

Moreover, DM2 patients have a lower risk of developing anesthesiologic complications, probably because of the less severe respiratory involvement (23).

On the other hand, posterior subcapsular iridescent cataracts are identical in DM1 and DM2 patients and laboratory tests showed the same pattern of hypogammaglobulinemia in DM2 and DM1, as well as elevated serum creatinine kinase and γ-glutamyltransferase, elevated follicle-stimulating hormone and low or low–normal testosterone levels in men, and diabetes (24).

CNS involvement was recently investigated in DM2 patients as well (24). Although cognitive impairment and white matter changes are more common in DM1, structural abnormalities such as generalized brain atrophy and white matter hyperintense lesions have also been reported in DM2 patients (25). Moreover, positron emission tomography and single photon emission computed tomography studies demonstrated hypoperfusion in the prefrontal, temporal and parieto–occipital cortex and basal ganglia (26), and the results of neuropsychological assessment indicated that several aspects of executive functioning are impaired to an equal extent in both DM1 and DM2 (27). A diffusion tensor imaging study by Minnerop et al. showed a reduction in grey matter volume in brainstem and adjacent hypothalamic and thalamic nuclei, along with a reduced corpus callosum thickness in DM2 subjects (28). Interestingly, in the literature atypical Parkinson's disease and levodopa-responsive early Parkinson's disease have been reported in two patients affected by DM2, respectively (29).

PATHOGENESIS OF MYOTONIC DYSTROPHY

The evidence of clinical and molecular similarities between DM1 and DM2 supports the hypothesis of a common pathogenetic mechanism related to the toxic gain of function of RNAs transcribed from the expanded alleles (30).

The mechanism by which this expansion leads to the clinical features is still unclear: the most reliable hypothesis suggests that RNA transcripts from the expanded allele create a gain-of-function mutation by inappropriately binding to the expanded CUG repeats in RNA transcripts of proteins involved in the regulation of correct splicing of RNAs transcribed from different genes (26).

Indeed, it has been documented that RNAs containing expanded CUG and CCUG repeats fold into hairpin structures and accumulate in nuclear foci (32-36), interfering with the activities of two specific RNA-binding protein families, the muscleblind-like proteins 1 (encoded by *MBNL1*) and 2 (encoded by *MBNL2*) (33, 37-39) and CUG-BP- and ETR-3-like factor 1 (CELF-1, encoded by the *CUGBP1* gene) (40). These two protein families antagonistically modulate alternative splicing of developmentally regulated genes, respectively inducing the adult or the embryonal expression pattern (41-44). Accordingly, an aberrant embryonal splicing pattern of a specific gene subset potentially involved in DM has been documented in tissues of DM patients and animal models of the disease (30), whereas transgenic models reproducing either the silencing of *MBNL1* (44) or the overexpression of *CUGBP1* (45) replicate some clinical features and aberrant splicing events observed in DM patients.

Indeed, in patients affected by DM1 and DM2, myotonia and insulin resistance seem to result directly from the spliceopathy of the muscle chloride channel protein 1 (ClC-1) and the insulin receptor, respectively (30). On the other hand, the exact relationship between other clinical features of DM and the molecular evidence of such an extended missplicing phenomenon, which until now is known to involve more than 20 genes (31), needs to be clarified further.

OPTIONS FOR TREATMENT

Therapeutic approaches to DM may be divided into two main categories: "symptomatic treatments" developed in order to reduce disabling symptoms, and "disease-modifying treatments", which attempt to modify the course of the disease by interfering with different targets in the molecular pathways involved in DM1. The former are currently used despite their slight and restricted efficacy over time, while the latter have recently been proposed, casting new light on the possibility of controlling the disease's progression.

Symptomatic treatments

Myotonia and muscle weakness are cardinal clinical features of DM and are often the first cause of a medical consultation for patients.

Myotonia

Myotonia is commonly thought to depend on altered functioning of muscle membrane channels, probably determined by alternative splicing of the chloride channels (46, 47). In general, sodium channel-blocking drugs are effective in reducing myotonia through the reduction of muscle membrane excitability. Antiarrhythmics (e.g., flecainamide, procainamide hydrochloride [48-50], mexiletine [51, 52], disopyramide [50, 52] and tocainide hydrochloride [52]), antiepileptic drugs (phenytoin [49, 52]) and quinine sulfate (48) have been reported to be successful in reducing myotonia. In particular, mexiletine hydrochloride has generally been considered to have the most favorable profile (53); preliminary data from two small randomized, double-blind studies have been presented, showing that it was safe and effective in treating myotonia in DM1 (54). However, definitive data have not yet been published.

Nevertheless, the main concern with the use of sodium channel-blocking agents is the potential risk for arrhythmias, which in turn are one of the main clinical disturbances in DM1. Furthermore, they can accentuate muscle weakness by reducing muscular action potentials (55).

Tricyclic agents (e.g., imipramine [56] and clomipramine [57]), calcium channel blockers (e.g., nifedipine [58]), benzodiazepines (e.g., diazepam [59]), taurine (60) and prednisone (48) have also been used to treat myotonia.

However, the usefulness and safety of medical treatments for myotonia is still debated, as recently confirmed by the negative conclusion of a Cochrane collaboration review based on 8 trials involving 103 DM patients (61).

Weakness and muscle wasting

Muscle wasting in DM1 is principally caused by an alteration in muscular anabolism, as suggested by studies assessing protein synthesis in affected cells (62). This observation stimulated the interest for anabolic therapies to counteract muscle wasting and, as a consequence, weakness.

Creatine monohydrate had no effect on muscle strength in DM1 (63, 64), but it reduced myalgia in DM2 (65). Testosterone administration did not achieve significant results (61).

A pilot study demonstrated that the i.v. administration of dehydroepiandrosterone (DHEA) was associated with improved muscle strength and myotonia in DM1 (67), providing the rationale for a larger multicenter, randomized clinical trial, results of which were recently published (68). The study was conducted in 75 adult DM1 patients receiving oral DHEA (100 or 400 mg) or placebo for 12 weeks. The authors reported that DHEA had no significant effect on muscular, respiratory or cardiological disturbances of DM patients.

The most efficient activator of muscular anabolism is insulin-like growth factor I (IGF-I). In 1995, Vlachopapadopoulou et al. reported that recombinant human IGF-I (rhIGF-I) efficiently reduced muscle weakness in DM patients (69). Nevertheless, the formulation used had a short circulating half-life, creating the need for a twice-daily subcutaneous injection and probably reducing the opportunity to observe a stronger clinical effect. Therefore, a new formulation of rhIGF-I, constituted by rhIGF-I complexed with IGF-I-binding protein 3 (rhIGF-I:rhIGFBP-3) was introduced. A phase II trial (NCT00233519) of this new formulation was conducted in 15 DM patients who received daily subcutaneous administration for 24 weeks. The first 6-patient cohort received 0.5 mg/kg/day rhIGF-I:rhIGFBP-3 for 8 weeks followed by 1.0 mg/kg/day rhIGF-I:rhIGFBP-3 for 16 weeks, and the second 9-patient cohort received 3 consecutive 8-week treatments with 0.5, 1.0 and 2.0 mg/kg/day. However, the results of this trial have not yet been published.

The regulatory mechanisms of muscle mass have offered other therapeutic opportunities. In recent years, the role of myostatin, a protein belonging to the transforming growth factor β (TGF-β) superfamily, has been thoroughly investigated. Myostatin plays a central

role in negative regulation of muscle growth (70) and has received great attention for its possible role in muscular diseases (71). Several strategies for the inhibition of myostatin have been developed in order to counteract muscle wasting. In 2002, Bogdanovich et al. (72) reported that myostatin-blocking antibodies were effective in a mouse model of Duchenne dystrophy. Nevertheless, after evaluation of clinical data, the development of humanized myostatin-directed antibodies (stamulumab, MYO-029) was discontinued in 2008 (71). Myostatin inhibition can also be obtained by means of its propeptide, one of its physiological negative regulators. Administration of myostatin propeptide was proven effective in mouse models of Duchenne dystrophy (73) and limb–girdle muscular dystrophy with calpain-3 (*CAPN3*) mutation (74). Other inhibitory strategies include small interfering RNAs (75), antisense oligonucleotides (AONs) (76) and TGF-β receptor inhibitors (77). To our knowledge, none of the myostatin inhibitors has been tested in DM1, although patients with prominent muscle wasting could probably benefit from myostatin blockade.

Treatment of systemic symptoms

As described above, DM is characterized by several disabling and potentially life-threatening systemic manifestations that deserve attention in patient management. Respiratory failure due to progressive muscular weakness is the main cause of death in DM patients (78-80). The current approach to this issue is the same as for other neuromuscular disorders, namely noninvasive ventilation at the onset of nocturnal hypoventilation and tracheostomy in patients who are unable to adequately protect their airways (81).

Cardiac involvement in DM is relevant, as sudden death occurs in a consistent proportion of patients (about 33% of total deaths [80]), mainly as a consequence of arrhythmias. In recent years, great attention has been directed toward clinical and instrumental predictors of malignant arrhythmias; clinical and genetic factors have been reported to be weak predictors of severe arrhythmias (82). On the other hand, Groh et al. (80) have reported that atrial tachyarrhythmia (i.e., sustained atrial tachycardia, flutter, fibrillation) and severe ECG abnormalities (rhythm other than sinus; P-R interval > 240 ms; QRS duration > 120 ms; second- or third-degree atrioventricular block) could be strong predictors of sudden death in DM1 patients. Subjects should receive pacemaker or implantable cardioverter according to current pacemaker guidelines (83).

As mentioned above, daytime sleepiness is one of the most common symptoms of DM1. Psychostimulants, such as modafinil, have been tested as treatment options. Three double-blind, randomized trials conducted on a total of 73 subjects with DM1 reported a positive effect of modafinil on daytime sleepiness, as assessed by the Epworth Sleepiness Scale, (84, 85), the Stanford Sleepiness Scale (85) or the Maintenance of Wakefulness Test (86). These findings were not replicated by Orlikowski et al. (87) in a multicenter study including 28 patients.

Disease-modifying treatments

The main pathogenetic mechanism of DM is currently thought to involve an RNA toxicity due to the interaction between the CUG-expanded (in DM1) or CCUG-expanded (in DM2) RNA and RNA-binding proteins, such as muscleblind-like proteins (44, 88) and CELF-1 (41, 89). Among the effects of CUG-expanded RNA there is a loss of muscleblind-like protein function and an increase of CELF-1, which act as antagonists in RNA splicing regulation (37, 88). In particular, CELF-1 promotes the inclusion of specific fetal exons in fetal and embryonic tissues during development, whereas muscleblind-like proteins (muscleblind-like protein 1, 2 and 3) induce a skipping of such exons, leading to the expression of adult isoforms. As described above, an alteration of this fine regulation can lead to some of the most important clinical features of DM. Thus, one therapeutic strategy would be to influence the levels and/or functioning of muscleblind-like proteins and CELF-1 in order to overcome this RNA-mediated toxicity.

An overexpression of *MBNL1* was obtained in a transgenic mouse model of DM (HSA^{LR}) using an adeno-associated virus (AAV)-mediated transduction via intramuscular injection (44). The authors hypothesized that increased muscleblind-like protein 1 availability would lead to a saturation of CUG expansion binding sites, restoring the correct splicing pattern. In fact, a twofold expression of *MBNL1* leads to a significant reduction of myotonia in injected muscles. This result was correlated with a reversion of the spliceopathy involving specific muscle-expressed pre-mRNAs affected in both human DM and the HSA^{LR} model, such as those of CIC-1, LIM domain-binding protein 3 (a striated muscle PDZ-LIM protein localized in Z lines), ryanodin receptor 1, sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) and fast skeletal muscle troponin T.

Analogously, de Haro et al. (90) reported that overexpression of human *MBNL1* in a fly model of DM1 was able to rescue the muscle degeneration and eye development disorganization observed in animals carrying a 480-CUG triplet expansion. Conversely, ocular and muscular pathology was increased in flies overexpressing *CUGBP1*.

However, the use of AAV has several potential limitations, mainly regarding the possibility of systemic delivery, although promising data on AAV gene therapy have been reported in Duchenne dystrophy (91). To date, AAV gene therapy has never been applied to humans affected by DM.

Since the main pathogenetic mechanisms of DM seem to be RNA-mediated, other interesting therapeutic strategies could involve the direct modification of RNA–protein interactions. Promising data are emerging from the application of AONs, short RNA sequences that interfere with the splicing of specific exons by steric block (92). The use of AONs may recover the production of proteins affected by the splicing alterations typical of DM.

Wheeler et al. (93) used systemic delivery of morpholino AON to correct the spliceopathy of muscle CIC-1 in the HSA^{LR} mouse model of DM. In DM, the fetal exon 7a is included during the post-transcriptional modifications of the mRNA, inducing a frame shift and a premature stop codon in exon 7. The administration of an AON directed toward 3′ and 5′ splice sites of exon 7 caused the skipping of exon 7a during splicing. The final effects of these changes were the recovery of normal density and function of CIC-1, and a reduction of myotonia as assessed by electromyography.

An alternative way to use AONs was proposed by the same group (94). They reported the effect of intramuscular delivery of a mor-

pholino AON (CAG_{25}) able to bind CUG-expanded RNA in a mouse model of DM. From the biochemical point of view, the administration of CAG_{25} determined the release of sequestered muscleblind-like protein 1, and expression and function of CIC-1 were rescued. Furthermore, CAG_{25} caused an increase in CUG-expanded RNA in the cytoplasm and a reduction of ~50% in CUG-expanded RNA levels, probably via the enhancement of its degradation due to breaking up of the RNA–protein complex.

Mulders et al. (95) reported data on a fully *2′-O*-methylphosphorothioate-modified $(CAG)_7$ AON able to silence mutant *DMPK* RNA expression, reducing the number of ribonuclear aggregates in a selective and CUG expansion length-dependent manner. When administered directly into muscle, this AON had a normalizing effect on aberrant pre-mRNA.

The use of AONs has several potential limitations; as for AAV, systemic delivery of AONs may be difficult. Studies in Duchenne dystrophy have shown that systemic delivery of AONs could be effective in manipulating gene transcription (96). However, in Duchenne dystrophy, the entrance of AONs in myocytes may be favored by the disruption of normal membrane architecture, which remains intact in DM1. More recently, Wheeler et al. (94) bypassed this limitation, inducing an increase of muscular membrane permeability by electroporation. Moreover, AON efficacy is restricted only to the specific mRNAs for which they are synthesized. Thus, the identification of other transcripts unequivocally involved in clinical features of DM1 is necessary to develop additional useful AONs.

Another important therapeutic strategy in DM1 is focused on RNA toxicity. In DM1 cells, the pathological RNAs are retained in the nuclear foci, sequestering RNA-binding proteins and exerting a toxic effect on myogenic differentiation and RNA splicing processes. Thus, one potential therapeutic approach is to get rid of the toxic RNA from cells. Indeed, RNA toxicity has been targeted directly using antisense gene therapy by Furling et al. (97). They reported the effect on muscle cells in culture of the transfection by an antivirus expressing antisense RNA, designed to degrade expanded *DMPK* transcripts. The cultured cells transfected with antivirus showed reduced levels of these *DMPK* transcripts, with improvement of fusion and differentiation, and, interestingly, a reduction of CELF-1 levels.

CUG-expanded RNA could also be cleaved by means of a ribozyme, a small catalytic molecule with RNA site specificity. Langlois et al. (98) reported that the administration of a ribozyme targeting the 3′-untranslated region of the *DMPK* mRNA to DM myoblasts caused a 63% reduction of the CUG-expanded transcripts, with a concomitant reduction in the number of *DMPK* mRNA-containing nuclear foci. The authors also reported a partial restoration of insulin receptor expression.

Krol et al. (99) assessed the effect of short interfering RNAs (siRNA) on the transcription of *DMPK* in DM1 cells. The authors transfected fibroblasts with a short sequence of RNA containing CAG repetitions, $si(CAG)_7$, obtaining a reduction of normal and mutant *DMPK* transcripts. They obtained similar results in Huntington's disease and spinocerebellar ataxia fibroblasts, demonstrating that the siRNA mechanism of action was quite specific for mutant genes. However, studies assessing the use of siRNA in DM animal models are not available to date. Hence, their potential beneficial effect must be further verified.

More recently, a new molecular target for disease-modifying therapy is represented by small molecules that are known to bind structured nucleic acid. In particular, pentamidine was recently demonstrated to disrupt binding of muscleblind-like protein 1 to CUG repeats. Specifically, in cell culture and in mouse models of DM1, pentamidine reversed the aberrant splicing of two pre-mRNAs affected in DM1 (100).

CONCLUSIONS

Recent advances in molecular biology have clarified the basic mechanism of organ damage in DM, providing preliminary evidence for the effect of RNA-mediated toxicity on organ-specific targets. More precise knowledge about these biological phenomena will raise hopes for treatments for DM that may not only be symptomatological, but also target the fundamental pathogenetic processes of the disease. In perspective, such acquisitions could provide the opportunity to halt (or even reverse) the progression of the disease, preventing disability and the consequences of the systemic manifestations of DM.

From this point of view, DM is an extraordinary model for other diseases caused by triplet expansion, such as Huntington's disease and spinocerebellar ataxias. Thus, it is conceivable that future development of therapeutic strategies for DM could be applicable to patients affected by these diseases as well.

DISCLOSURES

The authors state no conflicts of interest.

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