BEYOND BORDERS: MYOTONIC DYSTROPHIES – A EUROPEAN PERCEPTION

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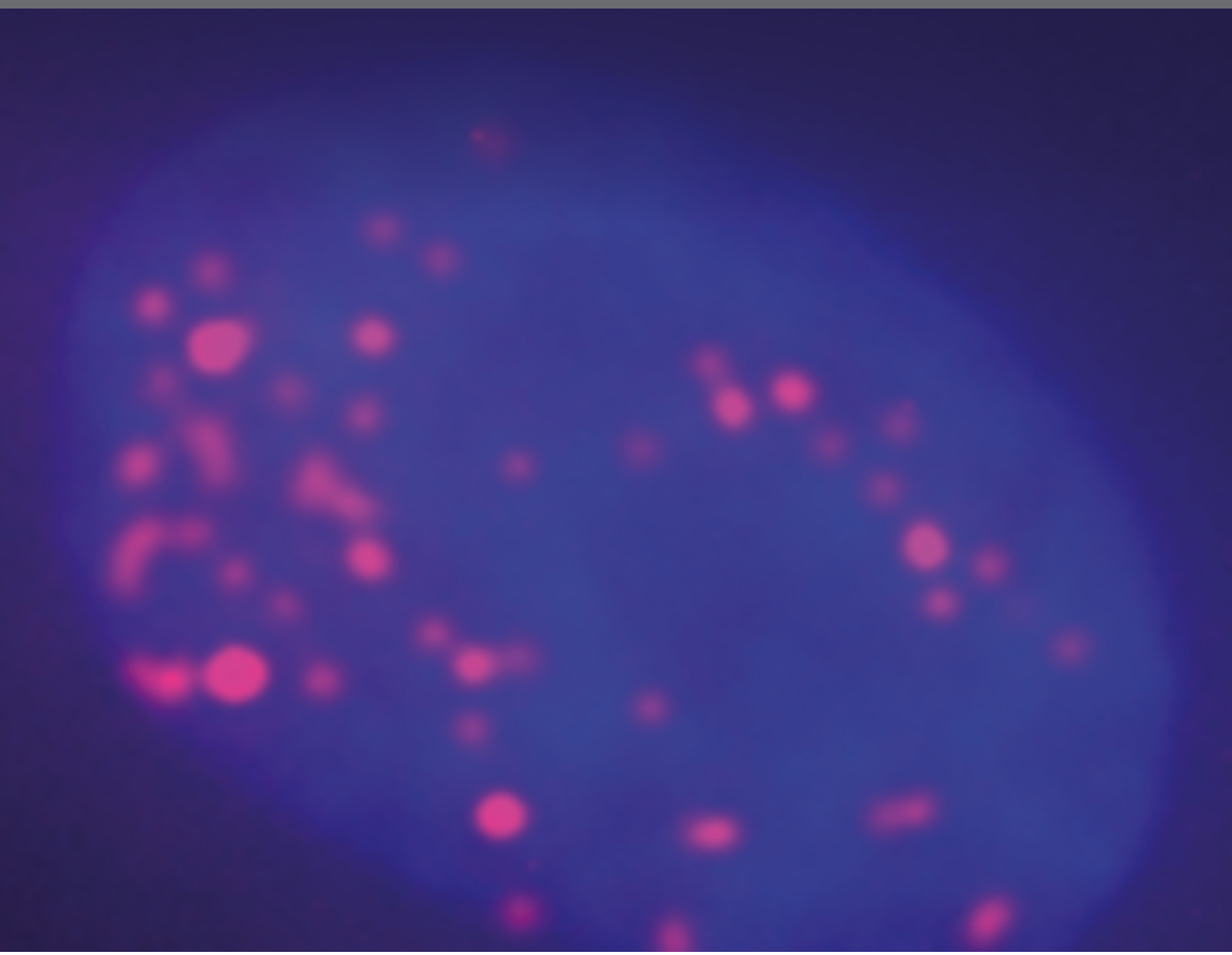

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BEYOND BORDERS: MYOTONIC DYSTROPHIES – A EUROPEAN PERCEPTION

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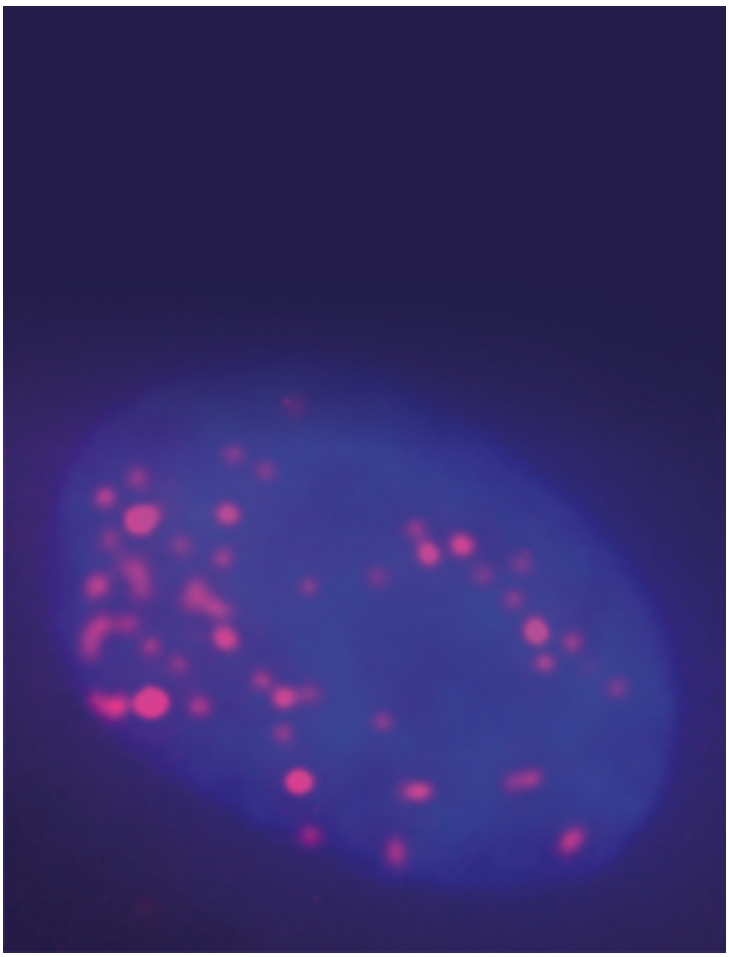

DM ribonuclear foci. Image: Benedikt Schoser.

Myotonic dystrophies (DMs) are pleotropic multisystemic diseases. These dominantly transmitted repeat disorders affect multiple organs of the human body at all ages – from the newborns to the elderly. The present Research Topic represents a timely addition to the expanding body of evidence which aims to provide novel perspectives in our understanding of myotonic dystrophies. This collection of original contributions

and standpoint reviews from multiple leading DM centres in Europe describes the state of the art for the characterization of the DMs diseases, the development of molecular strategies to target its multisystemic nature, and provides evidence of screening and testing novel therapeutic avenues.

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EDITORIAL
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Editorial: Beyond Borders: Myotonic Dystrophies–A European Perception

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Keywords: myotonic dystrophy type 1 and type 2, DM1, DM2, CNS involvement, repeat disorders, multisystemic diseases, cell models, animal models

Editorial on the Research Topic

Beyond Borders: Myotonic Dystrophies—A European Perception

Myotonic dystrophies (DMs) are pleotropic multisystemic diseases. These dominantly transmitted repeat disorders affect multiple organs of the human body at all ages—from the newborns to the elderly. DMs are highly inconsistent in terms of age at onset, severity of symptoms, and clinical patterns. Even within families, the onset and pattern of organ involvement remains enigmatic. Anticipation, with aggravation of the disease severity and earlier age at onset through successive generations, is particularly evident in DM1 that can affect adults and children at birth or during childhood 26 years ago, the identification of the DM1 repeat mutation in the DMPK gene on chromosome 19 opened the box for these diseases. The highly unstable CTG repeat expansion involved in DM1 usually increases from one generation to the next and is, to some extent, linked to disease severity. Clinically, patients with DM1 can be subdivided into five main classes, distinguishable by the prevalence of the presenting clinical pattern: congenital, childhood-onset, juvenile, adult-onset, and late-onset/asymptomatic. In myotonic dystrophy type 1 (DM1), the disease leads to a premature death, whereas in myotonic dystrophy type 2 (DM2) premature aging can be observed. In the past decades, much progress has been made in the pathomolecular understanding of the underlying DNA and RNA mechanisms of clinical miscellaneous DM symptoms. Presently, we are on the verge of transferring multiple bench-made molecular experimental therapies and knowledge into clinical therapeutic tools and reality, with the ultimate aim of alleviating and, eventually, curing the diseases. The present Research Topic represents a timely addition to the expanding body of evidence which aims to provide novel perspectives in our understanding of myotonic dystrophies. This collection of original contributions and standpoint reviews from multiple leading DM centers in Europe describes the state of the art for the characterization of the DMs disease, the development of molecular strategies to target its multisystemic nature, and provides evidence of screening and testing novel therapeutic avenues.

As an introduction to the current phenotype concept in myotonic dystrophy types 1 (DM1) and type 2 (DM2), Wenninger et al. summarize clinical core features of these highly variable subtypes.

Callus et al. focused on the neuropsychological and psychological assessment in a study on 31 DM1 patients. In 19.4% of DM1 patients a moderate or high level of symptoms intensity index is found. Fatigue and daytime sleepiness are associated with higher levels of psychoticism. Longer disease duration is associated with cognitive impairment evaluated through ENB-2 ($p < 0.05$). The need of neuropsychological and psychological screening and support for these patients and their families is addressed and a clinical protocol is advanced.

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Minnerop et al. summarize the clinically important neuroimaging data. Structural gray and white matter abnormalities are seen in both DMs. In type 1, a consistent widespread cortical and subcortical involvement of gray and white matter affecting the whole brain is observed. Spectroscopy studies show neuronal and glial damage in both types. Correlative analyses of neuroimaging and clinical parameters stay diverse and are not well-reproducible. Latest insights argue more for disturbed networks as functional and structural substrates of the clinical symptoms. Longitudinal studies are required for future therapeutic studies.

Mahyera et al. report an estimated DM2 prevalence of 9 in 100,000, being as prevalent as DM1 in Germany. The expanded DM2 CCTG repeat tract comprises not only CCTG tetraplets but also repeated TG dinucleotides and TCTG tetraplet elements as well as NCTG interruptions. The normal allele sizes in the German population reveal that the CCTG repeat tract is usually interrupted by at least three tetraplets which is supposed to render stability. The largest analyzed normal allele has 23 uninterrupted CCTGs and may represent an instable early premutation allele. Their diagnostic results support a premutation range between 25 and 75 CCTGs, however clinical relevance of these premutation alleles are still uncertain. A fluid transition of penetrance is more likely as a clear cut-off of CCTG numbers in DM2.

Meinke et al. reflect on accelerated aging in DM. Clinical DM features are similar to aging aspects. Therefore DM could be classified as a segmental progeroid disease. However, molecular parallelism of accelerated aging in DM and segmental progeroid disorders are not reported yet. Now on cellular level molecular similarities to some progeroid syndromes of the nuclear envelope are detected. This first clinico-cellular comparison claims for the qualification of DM as a true segmental progeroid disorder.

Braz et al. summarize the modeling of myotonic dystrophy in mice, in order to provide investigational tools of the molecular and cellular pathogenesis. Mouse models are contributing intensely to our disease understanding. However, we still do not know how the molecular abnormalities described translate into CNS dysfunction. The authors review mouse models for neuromuscular aspects of disease, therapy development, and describe current limitations.

Chakraborty et al. discuss the molecular basis of cardiac dysfunction in myotonic dystrophies. Drosophila combines the acquiescence of its invertebrate genetics with the possibility of quickly acquiring physiological parameters. They review cardiac issues in both DMs, and the cardiac toxicity of non-coding CUG (DM1) and CCUG (DM2) repeat RNA in flies. Overexpression of muscleblind manages to strongly suppress arrhythmias and fractional shortening causing the cardiac phenotypes in flies. Small molecules pentamidine and daunorubicin are able to rescue cardiac phenotypes. Consequently, an assessment of candidate therapeutics in flies is possible.

André et al. review the pleiotropic problems of development, growth, regeneration, and aging of skeletal muscle. The molecular and cellular processes and roles of embryonic and adult muscle-resident stem cells in growth, homeostasis, regeneration, and premature aging is updated. Progenitor cells from extramuscular sources, such as pericytes and mesoangioblasts, participate in myogenic differentiation and may be of therapeutical potential for DM.

Matloka et al. review how cellular models decipher the molecular basis of DM1 and describe currently available cell models, ranging from exogenous expression of the CTG tracts to variable patients' derived cells.

Finally, López-Morató et al. report that therapeutic strategies for DM1 are mostly been focused on targeting CUGexpDMPK via reducing their expression and/or preventing interactions with MBNL1. Antisense oligonucleotides targeted to the CUG repeats in the DMPK transcripts are of particular interest due to their potential capacity to discriminate between mutant and normal transcripts. Nevertheless, alternative strategies using small molecule chemicals acting independently of a direct interaction with CUGexpDMPK are also reported. They summarize these chemicals and describe the beneficial effects in DM1 models. Moreover, they present potential mechanisms of action of these compounds and pathways they affect which could be considered for future therapeutic interventions in DM1.

As guest editors for this research topic on "Beyond borders: Myotonic dystrophies—a European perception," we are delighted to commend to you the collection of 10 articles as an important contribution to the molecular and clinical medicine from Europe to the patients living with myotonic dystrophies.

AUTHOR CONTRIBUTIONS

BS and GM conceived the manuscript. BS drafted the paper. GM critically appraised and edited the manuscript. Both authors read and approved the final version of the paper.

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REVIEW
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Core Clinical Phenotypes in Myotonic Dystrophies

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Myotonic dystrophy type 1 (DM1) and type 2 (DM2) represent the most frequent multisystemic muscular dystrophies in adulthood. They are progressive, autosomal dominant diseases caused by an abnormal expansion of an unstable nucleotide repeat located in the non-coding region of their respective genes DMPK for DM1 and CNBP in DM2. Clinically, these multisystemic disorders are characterized by a high variability of muscular and extramuscular symptoms, often causing a delay in diagnosis. For both subtypes, many symptoms overlap, but some differences allow their clinical distinction. This article highlights the clinical core features of myotonic dystrophies, thus facilitating their early recognition and diagnosis. Particular attention will be given to signs and symptoms of muscular involvement, to issues related to respiratory impairment, and to the multiorgan involvement. This article is part of a Special Issue entitled "Beyond Borders: Myotonic Dystrophies—A European Perception."

Keywords: myotonic dystrophies, DM1, DM2, phenotypes, myotonia, sleep disorders, repeat expansion diseases

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INTRODUCTION

The genetic background of myotonic dystrophies type 1 and 2 (DM1 and DM2) is due to repeat expansions of unstable nucleotides in untranslated DNA regions causing mis-splicing of mRNAs, which affects almost all cells and organs of the human body. This sum of alterations leads to an extremely heterogeneous phenotype with musltisystemic involvement. Many findings and symptoms of DM1 and DM2 overlap, but important differences usually allow their prompt clinical distinction (**Table 2**). Both types of myotonic dystrophies represent the most common inherited muscle disorders in adulthood with regional variations in prevalence and incidence. In general, DM1 occurs more frequently than DM2, with some exeptions in northern and mid European countries such as Finland, Germany, and Czech Republic, where DM1 and DM2 are almost equally represented. **Table 1** provides a useful summary of the country-specific prevalences (1–4).

KEY ASPECTS IN MYOTONIC DYSTROPHY TYPE 1

For DM1, there is a rough correlation between the expansion of CTG-repeats and the onset of symptoms as well as the severity of the disease; nevertheless predictions about the clinical features and the progression of the disease based on CTG-repeat size should be made very carefully (23, 24). 5 to 37 CTG-repeats are physiologic in healthy individuals. An expansion between 38 and 49 repeats does typically not cause any symptoms and reflects the premutation phenotype (3). The following four phenotypes are based on CTG-repeat sizes and onset of symtoms. It is important that phenotypes and CTG-repeat sizes do not show a linear and strict relationship and thus may overlap (25, 26):

- a mild phenotype with an expansion of 50–150 CTG-repeats,
- a classic phenotype with a wide span from mild to severe symptoms and an expansion of 50–1,000 CTG-repeats,
- a childhood/juvenile phenotype with early-onset and typically >800 CTG-repeats, and
- the most severe "congenital form" with usually >1,000 CTG-repeats.

CTG-repeats will expand in every following generation, and fully penetrant alleles occur with >50 CTG-repeats. This results in the so called anticipation, a clinical term describing an earlier onset with a more severe phenotype in the next generations (26). Furthermore, the repeat instability leads notably to premature aging of almost all organs, so DM1 may be counted among the progeroid diseases (27). The most typical appearance of DM1 is the "adult-onset" or "classic" phenotype with a CTG-repeat size ranging from 50 to <1,000. It is characterized by a distinctive combination of muscular symptoms, such as facial weakness, ptosis, grip myotonia, and distal muscle weakness with muscular atrophy. The classic phenotype is typically accompanied by extramuscular symptoms like cognitive impairment, cataracts, and diabetes mellitus. Nevertheless, as this multisystem disorder often presents with a high variability, some patients may primarily show only non-specific extramuscular symptoms like fatigue, daytime sleepiness, gastrointestinal symptoms, or cardiac conduction defects in an early stage of the disease, which could delay the diagnosis. Mildly affected patients with CTG-repeat sizes 50–100 may have normal or only minimally shortened lifespan (28). Because of comorbidities, such as cardiac and pulmonary complications, life expectancy is, however, reduced in about 70% of the patients with the classic phenotype (25).

TABLE 1 | Country-specific prevalences of DM1 and DM2.

Country	Disease	Prevalence ($\times 10^5$)	Reference
Croatia	DMs	18.1	(5)
Czech Republic	DM2	DM2 > DM1	(6)
Finland	DM2	10	(7)
Finland	DM2	54	(8)
Germany	DM2	DM1 = DM2	(9)
Israel	DM1	15.7	(10)
Italy	DMs	2.1	(11)
Italy	DM1	9.3	(12)
Italy	DM2	0.9–1	(13)
Italy	DM1	9.6–11.7	(13)
Japan	DMs	9.1	(14)
Spain, Mallorca	DMs	10.8	(15)
New Zeland, Otago	DMs	11.6	(16)
North Ireland	DMs	11.9	(17)
North Ireland	DMs	34	(18)
North UK	DM1	10.4	(19)
Quebec	DM1	210	(20)
Serbia, Belgrade	DM1	5.3	(21)
Taiwan	DM1	0.5	(22)

SPECIAL ASPECTS IN MYOTONIC DYSTROPHY TYPE 2

DM2 (also referred to as proximal myotonic myopathy) is caused by the expansion of the tetranucleotide CCTG-repeat in the first intron of *CNBP* (cellular nucleic acid-binding protein), formerly known as zinc finger protein 9 (*ZNF9*) gene (29). Similar to DM1, these expansions are extremely unstable, causing widespread cellular abnormalities of mRNA splicing. In DM2, the expansion ranges from 75 to 11,000 with a mean of 5,000 CCTG-repeats. In contrast to DM1, there is no correlation between clinical phenotype and CCTG-repeat length and no anticipation has been observed (29, 30).

MUSCULAR SYMPTOMS

Muscular Weakness

The symptoms myotonia, muscular weakness, and muscular atrophy are the principal traits of DMs and gave the eponym for these two types of the disease. In DM1, patients present with characteristic distally predominant muscular atrophy and weakness mainly involving finger flexors, wrist flexors, and foot extensors (**Figures 1A,B**). The latter will cause foot drop and gait disturbance with repeated falls and injuries (31). In contrast to this, muscle weakness in DM2 is typically proximal and axial, affecting more consistently the neck flexors, hip flexors, and hip extensors (**Figure 1C**) (30, 32). This predominantly proximal muscular involvement has been documented also by MRI studies that showed an early degeneration of the erector spinae and gluteus maximus muscles (33, 34). Muscle weakness is one of the most frequently reported symptoms in DM1 (>45% of patients with adult phenotype) and

TABLE 2 | Core Clinical Symptoms helpful for differentiating DM1 and DM2.

		DM1	DM2
Age of onset		Depends on CTG-repeat-size, in common first symptoms earlier than in DM2	30–40
Family history		Increasing severity of symptoms throughout generations (anticipation)	Variability in symptoms, but no evidence for anticipation
General appearance	Head	Forehead balding	
	Face	Myopathic face, temporal wasting, ptosis	
	Bulbar	Frequent: nasal/slurred speech, dysphagia	In some cases: dysphagia
Muscle	Weakness	Distal	Proximal and axial
	Myotonia	Handgrip, tongue	mild proximal
	Atrophy	Distal, early	Proximal, late
	Myalgia	Not typical, but may be secondary due to regional muscle imbalance	Predominant
Sleep disturbances		Central sleep apnea, obstructive sleep apnea, respiratory muscle weakness	Central sleep apnea
Central nervous system	Daytime sleepiness	In almost every patient	Frequent
	Concentration problems	Frequent	In some patients
	Hearing impaiment	Rare in adults, more frequent in congenital DM	Frequent
Diagnostics	Electromyography	Myotonic discharges in clinically affected and not affected muscles	Proximal, but can be absent

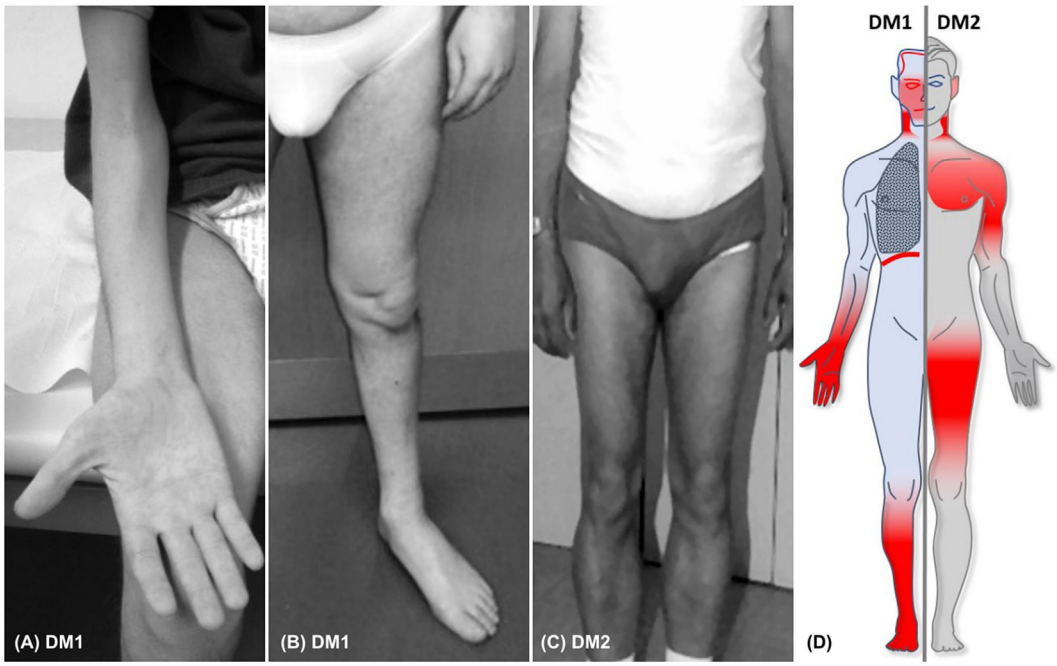

FIGURE 1 | **(A,B)** Typical distal muscular atrophy in patients with DM1. **(C)** Atrophy of proximal muscles in a patient with DM2. **(D)** Figure illustrating the core phneotypes of DM1 (left) and DM2 (right). Regions of muscular involvement (weakness and atrophy) are highlighted in red.

DM2 (40–55% of patients) (26, 32). **Figure 1D** illustrates the predominantly affected muscle groups of patients with DM1 and DM2 (see **Table 2** for differentiating DM1 and DM2).

The typical facial appearance of DM1 patients ("myopathic face"—"hatchet face") is a prominent and early feature and is caused by weakness and atrophy of facial muscles and ptosis that might give the false impression of a tired, sad, or emotionless patient (35). Balding of the forehead and atrophying of the temporal muscle are often seen (**Figure 2**) and completes the overall picture of a patient with DM1. Severe weakness of orbicularis oculi muscles cause not only ptosis but also insufficient eyelid closure with risk of recurrent conjunctivitis. This facial muscle involvement is usually not seen in DM2 patients, thus it may help in differentiating DM1 from DM2 patients (**Table 2**).

Especially in patients with DM1, the speech can be nasal and slurred, due to the weakness of oropharyngeal muscles, sometimes causing chewing and swallowing difficulties.

FIGURE 2 | The characteristic face of a patient with DM1: long face, temporomandibular wasting, balding forehead.

Myotonia

Myotonia is a more frequent symptom in DM1 mainly affecting the fingers (grip myotonia), the jaw, and the tongue (36). Clinically, a warm-up phenomenon is usually observed when myotonia improves with repeated contractions, which is mostly true for grip myotonia, but also for myotonia of the tongue and the jaw (37, 38). An increased excitability of muscle fibers is thought to be the cause for myotonia, leading to continuous discharges of repetitive action potentials after voluntary contraction or mechanical stimulation (39) in electromyography (EMG). These myotonic runs can be detected with EMG even in clinically unaffected muscles of DM1 patients, but can be rare or even be absent in DM2 (40). On a molecular basis, it has been suggested that myotonia is caused by mis-splicing of the chloride channel (*CLCN1*, ClC-1) due to misregulated MBNL1 and CUGBP1

(41). Recent studies have investigated the relationship between a central nervous system involvement and myotonia, suggesting that myotonia should no longer be considered as a solitary peripherally triggered muscular symptom (42, 43). In one fMRI study, higher cerebral blood oxygen level-dependent signals (BOLD) in specific primary and secondary motor areas were found during myotonia episodes. This was interpreted as a relationship between myotonia and high-order motor control areas (44). In another study, the severity of myotonia correlated with diffuse white matter alterations in specific primary and secondary motor areas (45). There are contradictory reports about the correlation between myotonia, grip strength, and CTG-repeat length (46, 47) for DM1 patients. Overall, grip strength correlates negatively with CTG-repeat length in most studies, but this is not necessarily true for myotonia. In one recent study, there was a statistically significant correlation between grip myotonia and CTG-repeat length, but this was not clinically meaningful and not predictive (47).

Myotonia seems to be usually mild to moderate or even absent in many DM2 patients, impacting only minimally their quality of life (36). However, its occurrence in different cohorts ranges between 24 and 75% (30, 32, 40). This variability is partly due to the discrepancy sometimes observed between a history of myotonia reported by patients and the clinical evidence of myotonic phenomenon, which is observed on neurological examination only in a minority of DM2 patients. Few patients may, however, display a severe myotonia and in some of these cases additional mutations in ion channel genes CLCN1 and SCN4A have been identified (48, 49). It is, therefore, advised to screen atypical cases with severe myotonia for mutation in these genes that act as phenotype modifier enhancing the myotonic phenomenon in DM2 (49). Particularly limb girdle myotonia is frequently neglected and underdiagnosed. With aging, the presence of myotonia gradually becomes less clinically relevant as it is overwhelmed by the gradually worsening of muscle weakness (32). This trend is also confirmed in studies assessing quality of life of DM2 patients, where significant predictors of worse QoL (quality of life) were older age, worse muscle strength, and higher level of fatigue (50).

Musculoskeletal Pain or Myalgia

Musculoskeletal pain or myalgia may be present in some DM1 patients, but is less frequent in comparison to DM2 (**Table 2**). However, with the progression of the disease, a muscular imbalance due to weakness may occur and secondary complications such as regional myofascial pain or joint pain syndromes may develop even in DM1. In DM2, about 60% of patients complain of diffuse myalgia. These are usually exercise-related and worsen in cold temperatures (33, 51). Some patients consider pain as the most disabling symptom of the disease also because of its poor response to common analgesics (7). The pathophysiological mechanism of myalgia in DM2 is yet to be elucidated, but it is probably related to specific molecular changes occurring in the muscles of DM2 patients (52).

For adult-onset DM1, the first muscular symptoms can become apparent in early adulthood, but some patients may exhibit subtle symptoms like grip myotonia, ptosis or slurred speech in childhood. Patients with classic DM1 are typically diagnosed at around 30 years, but mildly affected patients with CTG-length 50–100 may present solely some slight myotonia or cataracts and may have their diagnosis delayed until they are around 40 years old (28). The clinical onset of DM2 typically occurs later than DM1, around the third to fourth decade; it may, however, often go unrecognized for several years due to only mild or unspecific clinical symptoms like myalgia or muscle cramps.

Muscular Respiratory Symptoms

Respiratory muscle weakness will occur in a high percentage of the patients with DM1 in an early stage of the disease and chronic respiratory failure may develop (53). Expiratory muscles seem to be affected sooner than inspiratory muscles, resulting in early recurrent pneumonia due to a weak cough and insufficient airway clearance. The exact prevalence of respiratory insufficiency in DM1 is unclear because symptoms of nocturnal hypoventilation overlap with typical neuropsychological symptoms like fatigue, daytime sleepiness, and concentration difficulties (54). As both respiratory muscle weakness and cardiac symptoms account most for the reduced survival of the patients, repeated testing for early diagnosis is essential. A pure respiratory muscle weakness rarely occurs in DM2 and only about 6–15% of patients require non-invasive ventilation (55).

EXTRAMUSCULAR SYMPTOMS

CNS Symptoms

Fatigue, daytime sleepiness, and concentration difficulties are frequently reported symptoms in DMs. In DM1, cognitive deficits were initially attributed to a low IQ or mental retardation, but recent studies show that this assumption was wrong for a large cohort of patients and mainly applies for cases of congenital myotonic dystrophy (CDM). In fact, for the classic phenotype of DM1, neuropsychological deficits are as variable as muscular symptoms, and even recent publications about the correlation of CTG-repeat size and neuropsychological deficits show contradictory study results (56–58). There seems to be a correlation between diffuse brain alterations in primarily white and secondary gray matter, linking the DM1 to the group of brain disconnection disorders (59). Caso et al. investigated 51 DM1 patients and found a correlation between changes in brain white matter and cognitive impairment (60). Cerebral white matter hyperintensities have been observed in both DM1 and DM2 patients, especially in those older than 40 years, but their clinical and functional significance still remains unclear (61–63). In a recent study about the educational profile of a large cohort of young DM1 patients, no significant differences compared to the healthy population were found (35), assuming that cognitive and concentration disturbances may occur later in the course of the disease in the context of a variable premature cognitive decline, as suggested by the study of Modoni et al. (56). Mild cognitive and behavioral symptoms are also present in DM2 patients. In particular, altered visuo-spatial and executive functions, reduced attention and flexibility of thinking, avoidant behavioral trait, and depression have been detected in these patients (63, 64). In many cases, neuropsychological disturbances

jeopardize the ability to work and reduce the quality of life more than muscular symptoms.

Excessive daytime sleepiness, fatigue, and concentration difficulties may also be caused by central sleep disturbances or sleep apnea. Sleep-disordered breathing is one of the earliest manifestations and occurs in a high percentage of patients with DM1 (65), but overlapping symptoms of nocturnal hypoventilation and CNS symptoms may delay diagnosis and treatment. Chronic central sleep-disordered breathing has an impact on quality of life, morbidity, and mortality and should be assessed frequently in every patient with DM1 (54, 55, 66).

Until now, little is known about changes in CNS causing cognitive deficits and central sleep disrupted breathing. Almost every clinical study is conducted with the usually more affected DM1 patients, therefore data for DM2 patients are limited. On a molecular basis, MBNL1 and probably CELF may both be involved in CNS alterations, but little is known about molecular defects causing highly variable CNS symptoms in DM1 (42, 43). The above mentioned aspects lead to a discussion as to whether CNS dysfunction is caused by altered neurodevelopment, by neuro-dysfunction or by neurodegeneration within the definition of progeroid diseases (67). The hypothesis of a neurodegenerative disease is endorsed by findings of tau pathology and neurofibrillary degenerations, even if no correlations with CTG-repeat length were found (68). Overall, CNS dysfunction seems to be multifactorial.

Eyes

The most frequent, early and typical extramuscular manifestation is the occurrence of early-onset cataract, observed in about 50–60% of patients (30, 32, 69). A medical history of cataract surgery in combination with muscular symptoms often leads to the diagnosis of DM, even in mildly affected patients without any sign of muscular impairment (70–72). The mechanisms underlying the pathophysiology of cataract in DMs are still largely unknown. At first, a potential effect of the CTG-mutation on the expression of neighboring genes such as SIX5 was considered in DM1 (73). But recent findings showed that SIX5 knock-out mice develop the nuclear type of cataract and not the posterior subcapsular/cortical type that are commonly observed in DMs. In addition, SIX5 is not adjacent to the DM2 repeat expansion so that this mechanism could not explain cataracts in DM2. More recent studies of global transcription performed on samples of lens epithelium in patients affected by DM1, DM2, and controls, identified a high similarity as regards the pattern of gene expression between DM1 and DM2 and hypothesized that common molecular mechanisms should be involved in cataract formation probably involving interferon signaling pathways (74, 75).

Endocrine Symptoms

Endocrine dysfunctions such as diabetes, hypogonadism, and secondary hyperparathyroidism with decreased Vitamin-D levels are frequent in DMs and their occurrence increases with progression of the disease (76–78). A cross-sectional study on 68 DM1 patients showed at least one endocrine dysfunction in 44% at baseline and in 84% after 8 years (76). Diabetes mellitus, if not properly treated, may complicate and aggravate the clinical picture due to diabetic polyneuropathy with worsening of gait instability and distal weakness. Hyperparathyroidism may contribute to fatigue and muscle impairment (76). More rarely, abnormalities in growth hormone secretion and glucose intolerance may be observed (79).

Hearing Impairment

Some degree of hearing loss has been described in DM2 since its first description (80). A recent systematic study on 56 Dutch and French DM2 patients then demonstrated that a mild to moderate hearing impairment was present in about 60% of examined patients. It is mostly a cochlear sensorineural hearing impairment which may be interpreted as an early presbycusis (81), well fitting in the interpretation of DMs as premature aging diseases. Similar features of cochlear impairment have also been described in some studies on DM1 patients (82).

Cardiac Symptoms

In DMs, cardiac involvement is common. Cardiologic comorbidities include arrhythmias, atrial fibrillation, and conduction defects (e.g., AV-blocks) and often requires the implantation of pacemakers. Other infrequent manifestations are sudden death, heart failure, Brugada syndrome, ischemic heart disease, and mitral valve prolapse (83, 84). Dilated cardiomyopathies may also occur in some patients, but are not frequently found. Cardiac abnormalities in DM2 are similar to those observed in DM1 but occur less frequently. According to a recent observational case–control study on a large cohort of DM2/DM1 patients, it emerged that electrocardiographic abnormalities as PR > 200 ms and QRS > 100 ms were more frequent in DM1 (respectively, 31 and 48%) than DM2 patients (10 and 17%). Of those, 6 DM2 vs. 28 DM1 patients needed a pacemaker/implanted cardioverter (85). In the same study, echocardiography did not show any significant structural abnormalities but it was previously reported that a cardiomyopathy might occur in about 3% of DM2 patients. In DM1, the severity of cardiac involvement seems directly related with the size of CTG-expansion as recently studied by Chong-Nguyen et al. (9, 30, 85, 86). Atrial fibrillations and arrhythmias increase the risk of cerebral ischemia (87) and mortality and morbidity significantly depend on early cardiologic diagnosis and treatment (83).

Gastrointestinal Symptoms

Along with elevations of creatine kinase, elevations of AST and ALT are frequent in patients with DM (88). In some cases, liver biopsies are performed because of these elevated "hepatic" enzymes without retrieving any pathologic result. The elevation of gamma-GT is suggested to be caused by contractions of bile canaliculi and bile ductules, whereas elevated levels of AST and ALT have their origin in skeletal muscle and go along with elevations of creatine kinase (89).

Alternating constipation, pseudo-constipation, bloating, and diarrhea are frequently reported symptoms in DM1, accompanied by stomach cramps, reflux, and regurgitation. They are caused by involvement of smooth and striated muscles and endocrine dysfunctions (90, 91). Swallowing problems are typical for DM1 patients and due to reduced oral transport that is caused by myotonia and weakness of the tongue. Dysphagia is caused by

reduced swallowing reflex and reduced esophageal motility (92) which causes the major clinical problem by risk of aspiration. In conjunction with weakness of early affected expiratory muscles, this results in recurrent pneumonia and increased risk of death. A reduced or absent gastrointestinal peristaltic movement was earlier shown in radiological studies as well as delayed intestinal transits (93). Megacolon with the risk of ileus, volvulus and rupture, is a significant and life-threatening complication. Delayed emptying of the gall bladder may increase the risk for gallstones.

Cancer

A higher incidence for neoplasms was found in several studies (28, 94, 95), most of them showed a predisposition in patients with DM1 for cancers such as skin cancer (like benign calcifying cutaneous tumors, pilomatricomas), thyroid, testicular, and prostate cancer. Because of the limited number of high-quality surveys and studies about the prevalence of cancer in DM1, further research is needed. A survey from the UK DM registry showed that 12.4% of the DM1/DM2 patients reported at least one benign tumor and 6.2% reported at least one malignant tumor with a high incidence of skin tumors (96), but there was no epidemiologic correlation with a non-DM-population.

Peripheral Polyneuropathy

There is some debate as to whether peripheral neuropathy is a multisystemic manifestation of DMs or are caused by metabolic and endocrine dysfunctions. Its manifestation is not typical at early stages of the disease but may occur in about one-third of patients in later stages (97) of DM1 patients and contributes to balance impairment and increased risk of falls (31, 98). There were no significant correlations between age, duration of neuromuscular symptoms or CTG-repeat size (98, 99), suggesting that the affection of peripheral nerve system is secondary to metabolic and endocrine dysfunctions.

CONGENITAL MYOTONIC DYSTROPHY (CDM)

Patients with congenital DM1 have large CTG-expansions of more than 800, usually around 1,000. Characteristically, these large expansions are caused by maternal transmission, but CDM with paternal transmission is also known (23, 100–102). Clinically, CDM patients are severely affected and symptoms are often present before birth as polyhydramnios and reduced fetal movement. Hypotonia, generalized weakness, hyporeflexia, bilateral talipes, contractures, arthrogryposis, facial dysmorphia (carp mouth, ptosis, long neck and face, temporal muscle atrophy), and a weak cry are typical symptoms at birth or in the first days after delivery. Weak sucking and respiratory insufficiency often make ventilatory support unavoidable. Respiratory insufficiency is present in about 50% of newborns and is the main cause of dramatically reduced survival with a mortality rate of 30–40% (103). Infants who survive will typically reach their motor and cognitive milestones with some delay but might be able to walk independently. Similarly to DM1, a distal weakness is typical in CDM and a proximal involvement indicates a poor prognosis (104). Besides muscular symptoms, cognitive impairment, and neuropsychological disorders are the most common and variable manifestations in CDM. Symptoms range from intellectual impairment to selective cognitive impairment, apathy, and autism, as well as impaired attention, severe anxiety, and mood and depression syndromes (3, 102, 105–107). In the course of the disease, patients might require special schooling. In their third and fourth decades, patients may develop secondary complications, such as severe contractures, scoliosis, and worsening of cardiorespiratory symptoms (4).

CHILDHOOD/JUVENILE ONSET DM1

The childhood and juvenile onset DM1 echoes the broad overlapping spectrum of symptoms of the congenital and the adult phenotypes. Commonly, there is an expansion of CTG-repeats of more than 800 repeats. First clinical symptoms may become apparent at age 1–10 for childhood onset and at age 10–20 for juvenile onset (3). Neurocognitive symptoms such as learning disability and learning difficulties are often prominent at age around 10 years and may become earlier apparent than muscular symptoms (107). In contrast to CDM, prenatal abnormalities or muscular symptoms right after delivery (neonatal hypotonia, sucking and swallowing difficulties and secondary dysmorphic features) are not typical, but a mild facial weakness or subtle facial dysmorphia may occur (3, 108). Early motor development is normal or only slightly delayed. Principal complaints in early childhood are speech and learning difficulties because of a mental handicap. At school, learning difficulties may become apparent and sometimes require special education. A study on 28 childhood-DM-patients showed that the full-scale IQ was significantly decreased (73.6) and 68% of the patients had repeated at least one school grade. 54% had additional psychiatric symptoms such as anxiety disorder, mood disorder, and attention-deficit-hyperactivity disorder (107). In adolescence, patients may show typical muscular and non-muscular symptoms of adult-onset DM1, e.g., like distal weakness, clinical myotonia, or gastrointestinal symptoms. Cardiologic symptoms, such as cardiac arrhythmias or cardiomyopathy, may occur, also leading to severe complications and sudden death (83). Life expectancy is not necessarily reduced, as long as core symptoms are recognized and treated sufficiently.

CONCLUSION

Myotonic dystrophies represent the most variable clinical phenotypes, so treatment stratification is key for any modern therapeutic approach. We still need much more understanding of the signs and symptoms of DM patients in correlation to their molecular origins.

AUTHOR CONTRIBUTIONS

SW: review of publications, writing, critical revision of manuscript for intellectual content, and final approval of the manuscript. FM: review of publications, writing, and critical revision of manuscript for intellectual content. BS: critical revision of manuscript for intellectual content.

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Neuropsychological and Psychological Functioning Aspects in Myotonic Dystrophy Type 1 Patients in Italy

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Introduction: Myotonic Dystrophy Type 1 (DM1) is an autosomal dominant genetic illness, characterized by a progressive loss of strength. Important deficits in cognitive functioning and a significant prevalence of psychiatric disorders have been previously reported.

Methods: A neuropsychological and psychological assessment was carried out in 31 DM1 patients (61% males) in order to measure the cognitive functioning and explore their personality profiles. The MMSE Mini-Mental State Examination, Frontal Assessment Battery (FAB), ENB-2 Battery assessing memory (short term, long term and working memory), integration capacities, visual-spatial ability, attention (selective, divided, shifting/switching) executive functions, praxis, discrimination and logic capabilities and psychopathology Symptom Check List 90-R (SCL-90-R) were administered. The neuropsychological and psychological evaluation of DM1 patients was carried out taking into consideration the clinical parameters (CTG repeat, age at onset, disease duration, Muscular Impairment Rate Scale (MIRS), Medical Research Council Scale (MRC) and the Epworth Sleepiness Scales (EPS)).

Results: Regarding psychopathology 19.4% of patients scored a moderate or high level of symptoms intensity index (GSI), 12.9% reported a high number of symptoms (PST) and 16.1% reported a high intensity level of the perceived symptoms (PSDI). Fatigue and daytime sleepiness resulted as being associated with higher levels of psychoticism (PSY). Only 1 patient reported a severe impairment in the spatial and temporal orientation, memory, language, praxis, attention and calculation. Longer disease duration was also associated with cognitive impairment evaluated through ENB-2 ($p < 0.05$).

Discussions and Conclusions: There are indications of the utility of neuropsychological and psychological screening and support for these patients and their families due to the link between disease duration and cognitive performances. A proposal of a clinical protocol, with an illustration of a clinical case report of a family is presented.

Keywords: myotonic dystrophy, neuropsychological assessment, psychological assessment, neuropsychological functioning, psychological funtioning, patient empowerment

INTRODUCTION

Myotonic dystrophy type 1 (DM1; OMIM #160900) is a multisystemic neuromuscular disorder, which represents the most common form of muscular dystrophy in adults (1). DM1 is an autosomal dominant disease caused by an expansion of unstable CTG repeats located within the 3′-UTR of the *dystrophia myotonica protein kinase* (DMPK) gene. Mutant transcripts contain the triplet repeats form RNA hairpins that accumulate as foci in cell nuclei (2–4). These toxic transcripts are thought to sequester alternative splicing regulators leading to splicing defects that are considered the primary cause of DM1 symptoms (5). DM1 patients suffer from muscle wasting, weakness, and myotonia but also from heart conductions defects and central nervous system alterations (1). An important characteristic of DM1 that can influence adults and children at birth (congenital DM1, CDM) or during childhood is anticipation, with the aggravation of the disease severity and onset at an earlier age through successive generations. Five main categories in which DM1 patients can be divided into have been identified by the IDMC—International Myotonic Dystrophy Consortium (6); "congenital, childhood-onset, juvenile, adult-onset, and late onset/asymptomatic." In each one of these five forms, specific clinical features and management problems are described, and there is a distinction from each other based on the prevalence of the main symptoms and apparition profiles.

It is well-known that brain involvement results in cognitive and psychiatric dysfunction, which have an important impact on patient quality of life (QoL) (7–9). Neurocognitive dysfunction such as intellectual disabilities, reduced IQ-values, speech and language delay, deficit in visuospatial and/or visuo-constructive skills is a hallmark of the childhood forms of DM1 (infantile and juvenile onset forms) (8, 10–14). This dysfunction is comprehensive of neuropsychiatric problems, such as predominantly inactive subtype of attention deficit hyperactive disorders or autism spectrum disorders (11, 12). The juvenile form is characterized by school and problems with peers and is often under-recognized. These childhood forms of DM1 have to be considered as a central nervous system (CNS) disease rather than a muscular or systemic disease (15). In the adult-onset form of DM1, several studies have described selective impairments in executive function, visuospatial function, processing speed, and attention (8, 16–18). Characteristic personality patterns and emotional disturbances including fatigue, daytime sleepiness, depression, and apathy have also been reported in DM1 patients (19–21). DM1 patients have a clear age-related decline of cognitive functions as demonstrated through detailed neuropsychological studies, linguistic levels, praxis evaluations, and executive task evaluations. Two recent longitudinal studies have reported a cognitive decline especially for verbal memory, attention, visuo-spatial construction, and processing speed (22, 23) reinforcing the previous observation of Sansone et al. (17) who observed a progression of frontal cognitive impairment. The level of decline does not tend to correlate with either the number of CTG repeats or the severity of muscle weakness (16, 22, 23). These studies give support for proposals on a possible degenerative brain process (24). Recently, a review on central nervous system involvement, highlighted the wide phenotype and variable cognitive involvement that may occur in myotonic dystrophies (25).

In a recent study aimed to describe the psychological characteristics of a large cohort of patients with DM1 in Canada, it has been reported that DM1 patients are at high risk of developing a psychiatric disorder (26). Moreover, psychological traits differ across phenotypes, with the most severe phenotype tending to show more severe psychological symptoms. High levels of anxiety and low esteem are associated with lower levels of education and higher repetition of CTG (26). The literature about DM1 patient population highlights the predominance of paranoid together with aggressive traits (27). Furthermore, a clinically significant personality impairment, with dependent and paranoid personality patterns has been found and this, in turn, may decrease QoL (28).

Advanced MRI studies demonstrated across the brain a widespread white matter disruption and a multifocal gray matter volume loss by using various single MRI techniques, including diffusion tensor imaging and voxel-based morphometry, with correlations found between corresponding quantitative MRI parameters and triplet expansion, neuropsychological tests, and the severity of muscular involvement (24, 29–37). Abnormal patterns of brain connectivity have also been reported in DM1 patients and have been demonstrated to account for patients' personality traits (38–40). A study of neuroimaging of brain in children and adolescents suggests a relationship between white matter damage and working memory (34).

Currently there is still no comprehension regarding the links existing between pathomolecular mechanisms, genetic abnormalities and CNS involvement. It is vital to identify the adequate biomarkers of brain involvement in DM1 as CNS outcome measures are needed for upcoming gene therapy clinical trials (19, 25, 41, 42).

To date there is no cure or specific treatment for DM1 and treatment is aimed at managing symptoms of the disease, such as Mexiletine for myotonia and Modafinil for excessive daytime sleepiness. However, new therapeutic approaches based on pathogenic mechanisms at RNA or DNA level may become feasible in the near future

Currently, the best that can be done for DM1 patients is to give them the best care and clinical follow up, merging it with psychological assistance and support in order to prevent alienation. It is well-established that chronic diseases present challenges to health-related quality of life, an individual's perceived physical and mental well-being. Rare diseases may also create additional threats to quality of life due to poor access to information, treatment and support, combined with high levels of stigma. Psychotherapy can help not only this kind of patients, but also their families, to learn new behaviors and ways of coping with the symptoms of the disease.

In 2012 the OPTIMISTIC study (Observational Prolonged Trial In Myotonic Dystrophy type 1 to Improve Quality of Life–Standards, a Target Identification Collaboration), a project founded by the European Commission, coordinated by the

Netherlands and involving collaborative partners from France, Germany and United Kingdom, was launched (43).

It was a multi-center, randomized trial, designed to evaluate, as primary objective, the effect and the maintenance of a tailored behavioral change intervention comprising cognitive behavioral therapy including graded exercise training against standard patient management on participation for severe fatigue, as measured by the DM1-Active scale (Rasch-built measure of activity and participation for DM1 people). This innovative study involved over 200 patients across Europe and represented the first project extending psychotherapy to a large cohort of DM1 patients. The intervention also involved caregivers.

All the studies carried out until now, give a strong indication that in DM1 patients, cognitive impairments and psychological problems can affect their behaviors toward medical providers and their access to appropriate care, leading to worse QoL. Thus, it appears that DM1 patients could obtain benefits improving patient support, especially social and psychological support, and by improving communication with patients.

It has been observed in the medical settings that patients often require more information than they are given from health professionals, and they feel that they would like to be more involved in therapeutic procedure (44). In order for an effective involvement of patients in their care and their increased engagement, it is essential to enact strategies to improve their health literacy and put them in a condition that they feel they have control on their disease (45). For this to occur it is also important to consider the patient's preferences and needs in the medical decision-making process (46).

In fact, it is also important to take into consideration the mental dimensions, which make all patients unique, along with the assessment of their biological and clinical characteristics, in order to be able to tailor the interventions to them (47). The more there are efforts to create a patient profile which takes into consideration as many psychosocial elements as possible, the more it is possible to give a personalized approach in healthcare, improving and facilitating the communication between the physician and the patient (48).

In order to be fully effective, personalized medicine needs patients to be engaged and informed about their condition, who are also encouraged to discuss what their various treatment options are and their consequence (49).

The main objective of our study is to carry out a neuropsychological (through ENB-2 battery, MMSE, FAB) and psychological assessment (through SCL-90-R) of patients diagnosed with DM1 in order to measure the cognitive functioning and explore the personality profiles of these patients (50) in order to have a general picture of the cognitive and psychological functioning of Italian DM1 patients. Based on the outcomes, a secondary objective is to envisage a neuro-rehabilitation and psychological support program.

On the basis of the Outcomes Measures in Myotonic Dystrophy type 1 (OMMYD-1) for our study we used neuropsychological tests and self-report symptom questionnaires that found consensus among scientific community and measure the same abilities (19)[1]. Based on the Canadian experience (26), the Clinical Psychology Service of IRCCS Policlinico San Donato has set up an assessment protocol aimed to describe the neuropsychological and psychological characteristics of DM1 patients and to evaluate and create a neuro-cognitive rehabilitation and psychological support service for the patients in order to preserve the cognitive reserve. The protocol has been designed to be administered in a time frame compatible with patient hospitalization or with their outpatient access to our Neuromuscular Center.

Due to the limitations of the sampling size, in this study the correlations between cognitive/frontal functioning and demographic, medical or psychological symptoms will be explored. This data will be used to better tailor the psychological and neuropsychological program which was created for the patients and their families

The assessment protocol has been tested on a small cohort of Italian DM1 patients and the psychological support program has been tested on a family with the father and two sons diagnosed with DM1 and the mother not affected by DM1.

MATERIALS AND METHODS

The authors analyzed the theoretical background in the current literature on Myotonic Dystrophy in order to identify the state of art of patients' neuropsychological and psychological characteristics and to design an efficient protocol to be used in this study.

We conducted a review of current literature using the methodology explained in **Appendix 1**. The aim was the analysis of the available neuropsychological and psychological researches on DM1 patients and consider a plot of studies, which are presented in **Table 1**.

Our research contains an original protocol that investigates both cognitive and psychopathological domains. The selection of instruments was based on the review of the current available literature which was previously described (see **Table 1**). Our study focused on the neuropsychological and psychological aspects of DM1 population. Most of the previous studies focused on cognitive functioning and its association with the medical characteristics of the disease, such as disease form or CTG length (12, 14–16, 18, 22, 23, 27, 30, 40, 50, 53, 58–60, 64, 67–69, 71, 72, 74, 75, 78) and other studies looked at the psychological characteristics (7, 12, 21, 26–28, 40, 50, 52–54, 56–63, 68–70, 73, 74, 77, 78). The purpose of this research was to investigate both cognition and a psychological symptomatology association. We opted for a battery containing screening tests (MMSE, for a general cognitive screening; FAB for the assessment of the executive functions) and we decided to go for ENB-2 battery vs. WAIS, because it is validated on the Italian population and it offers the advantage of a shorter assessment time (7, 11, 12, 14,

[1]During the OMMYD-1 Workshop, four neuropsychological tests have been found as relevant in a clinical trial. These tests are Trail Making Test A and B, Stroop Color and Word Test, FAS (oral word association), and WAIS-R Block Design subtest. In our ENB-2 battery, similar tests were used to challenge the same cognitive areas.

TABLE 1 | Review of neuropsychological and psychological literature on myotonic dystrophy.

Article	Country	DM form	Sample	Investigation areas	Materials
Angeard et al. (11)	France	Childhood form	24 DM1	Neurocognition in childhood	MIRS WAIS-III
Bajrami et al. (51)	Turkey	All forms	13 DM1	Cognitive functions and cerebral involvement	MRI scans MIRS MMSE (*Mini-Mental State Examination*) MoCA (*Montreal Cognitive Assessment*) EHI (*Edinburgh Handedness Inventory*) WAIS Buschke Selective Reminding test (neuropsychological) FAB (*Frontal Assessment Battery*)
Baldanzi et al. (52)	Italy	Adult-onset form	65 DM1, 26 healty controls	Disease's awarness	MIRS MRC (*Medical Research Council scale*) TIB (*Brief Intelligence Test*) BDI-II STAI-Y2 AES (*Apathy Evaluation Scale*) INQoL (*Individualized Neuromuscular Quality of Life Questionnaire*) Neuropsychological cognitive battery: Immediate and Delayed Recall (IR, DR) RAVLT IR, DR of Rey-Osterrieth Complex Figure (ROCF) Digit span and CBT (*Corsi's block test*) TMT A, B Stroop Test FAS FAB WSCT Raven's progressive matrices (PM47)
Bertrand et al. (26)	Canada	Mild and adult-onset	200 DM1 (152 adult-conset form, 48 mild-compromission/late-adult)	Psychological characteristic	SCL-90-R (*Symptom Check list-90-Revised*) MIRS (*Muscular Impairment Rating Scale*) NEO-FFI (*Revised NEO Personality Inventory*) Rosenberg scale (self-esteem) ASIQ (*Adult Suicidal Ideation Questionnaire*) WAIS-R (*Wechsler Adult Intelligence Scale-Revised*)
Bird et al. (53)	USA	Adult-onset form	29 DM1	Cognitive functions and psychological characteristics	WAIS Shipley-Hartford Scale [pure verbal misure of intellectual functions] MMPI (*Minnesota Multiphasic Personality Inventory*)
Bungener et al. (54)	France	All except for congenital form	15 DM1, 14 healty controls, 11 with FSHD	Psychopathology	Semistructured interview DSM-III MADRS (*Montgomery and Asberg*) HDRS (*Hamilton depressive scales*) Covi and Tyrer anxiety scales AT (*Abrams and Taylor scale for emotional blunting*) EHD (*depressive mood scale*) PAS (*the physical anhedonia scale*) SAS (*social anhedonia scale*)
Cabada et al. (55)	Spain	Adult-onset form	42 DM1, 42 healthy	Cerebral involvement, cognitive functions and psychopathological traits	Diffusion tensor imaging WML (White Matter Lesions) MRI Digit Span TMT-B WAIS-IV (Letters and numbers) Cubes of the Barcelona Test Revised CPT (Continuous Performance Test) Benton Visual Retention Test TAVEC (Verbal learning Spain Complutense Test) Interview for Deterioration in Daily Living Activities in Dementia Dysexecutive Questionnaire HARS BDI-II SCL-90-R

(Continued)

TABLE 1 | Continued

Article	Country	DM form	Sample	Investigation areas	Materials
Caso et al. (30)	Serbia	Juvenile and adult-onset form	52 DM1, 34 controls	Cognitive impairment and cerebral damage	MRI scans ACE-R (*Addenbrooke's Cognitive Examination–Revised*) Raven progressive matrices WAIS Digit symbol coding Arithmetic subtests TMT WCST WAIS digit span ACE-R memory subscore RAVLT ROCF ACE-R (*Addenbooke's Cognitive Examination*) visuospatial subscore ROCF Copy Test WAIS block design VOT (*Hooper Visual Organization Test*) ACE-R language subscore BNT (*Boston Naming Test*)
Colombo et al. (56)	Italy	Severe form	40 DM, 20 healty controls	Neuropsychological and psychiatric test	MMSE WAIS-R SADS SRT (*Symptom Rating Test*)
Cuthill et al. (57)	Canada	Not specified	13 DM	Depression and anxiety	HAM-A (*Hamilton Anxiety Rating Scale*) HAM-D (*Hamilton Depression Scale*) SDS (*Zung Self Rating Scale*)
Douniol et al. (12)	France	Childhood form	28 DM1	Cognitive functions and psychological symptoms: hyperactivity, autism, depression, anxiety, alexithymia, impulsivity; sleepiness	WISC-R or WAIS the SAMUEL (visual–spatial construction abilities) MINI (*Mini-International Neuropsychiatric Interview*) the Dominic-R the whole ADHD section of the Diagnostic Interview Schedule for Children IV or Adult ADHD Self-Report Scale ASME (*Autism Mental Status Examination*) Children's Depression Inventory or BDI Spielberger State-Trait Anxiety Inventory Toronto Alexithymia Scale Lecendreux (for children) or Epworth (for adults) scales Eysenck Impulsivity self-report questionnaire
Echenne et al. (15)	France and Canada	Congenital, infantile/juvenile form	32 DM1	Longitudinal study over cognition	WAIS
Ekstrom et al. (58)	Sweden	Congenital and childhood form	57 DM1	Autism in childhood forms	ADI-R (*Autism Diagnostic Interview-Revised*) FTF (*The Five to Fifteen*) [to assess ADHD] SCQ (*Social Communication Questionnaire*) Griffiths Mental Development Scales, WAIS or WISC
Ekstrom et al., (14)	Sweden	Congenital and childhood form	55 DM1	Cognition and adaptive skills	Griffiths Mental Developmental Scale WPPSI-R or WISC-III or WAIS-III VABS (*Vineland Adaptive Behavior Scales*)
Franzese et al. (59)	Italy	Juvenile and adult-onsetform	28 DM1	Cognition and personality	Bender Visual Gestalt Test Wechsler-Bellevue Cancellation task MMPI Irritability-Depression-Anxiety scales of Snaith, Constantopoulos, Jardine, and McGuffin

(Continued)

TABLE 1 | Continued

Article	Country	DM form	Sample	Investigation areas	Materials
Fujino et al. (7)	Japan	All except congenital form	60 DM1	Cognition and quality of life	MMSE WAIS-III VPTA (*Visual Perception Test for Agnosia*) [Story telling subtest] CAT (*Clinical Assessment for Attention*) WCST FAB TMT CAT Position Stroop test Fluency test Apathy Scale ESS PHQ9 (*Patient Health Questionnaire-9*) MFI (*Multidimensional Fatigue Inventory*) SRS (*Social Responsiveness Scale*) MDQoL (*Muscular Dystrophy QoL scale*)
Gallais et al. (22)	Canada	Adult or late-onset form	115 DM1	Cognitive decline in longitudinal study	MIRS WAIS-R CVLT (*California Verbal Learning Test*) RCFT Verbal Fluency BNT SCWT Ruff 2 and 7 Selective Attention Test
Gaul et al. (60)	Germany	Juvenile, adult and late-onset form	21 DM1, 21 DM2	Cognition in DM1 and DM2: depressive symptoms and fatigue; visuo-construction; attention; prefrontal functions	Profile of Mood States RCFT Symbol Digit Modalities Test VF (*Verbal fluency*) CAL (*Conditional-associative learning*) IGT (*Iowa Gambling Task*)
Goossens et al. (61)	Netherland-Belgium	Juvenile	24 DM1	Psychological characteristics in childhood	WISC-R CBCL
Jacobs et al. (62)	Belgium	Juvenile form	27 DM1	Cognitive functions and psychopathological	WAIS-III ASEBA (*Achenbach System of Empirically Based Assessment*) questionnaire about behavior checklist: YSR, ASR, CBCL and ABCL
Kalkman et al. (63)	Netherlands	Adult-onset form only	79 DM, 65 FSHD and 73 HMSN-I patients	Psychiatric disorders in neuromuscular disorders	SCID-I-R (*structured clinical interview for DSM-IV axis I*) BDI SCL-90 GHQ-12 (*General Health Questionnaire-12*) MRC CIS (*Checklist Individual Strength*)
Kleberg et al. (64)	Sweden	Juvenile, adult-onset and late-onset form	33 DM1, 30 healty controls	Cognitive functions and facial memory	RBMT-E (*Rivermead Behavioural Memory Test*) Neuropsychological tests: Block design test Vocabulary test RAVLT RCFT
Laberge et al. (65)	Canada	Adult-onset form	27 DM1	Sleepiness	ESS (*Epworth Sleepiness Scale*) DSS (*Daytime sleepiness Scale*) CFS (*Chalder Fatigue Scale*)
Malloy et al. (66)	USA	All forms	20 MMD, 20 normal control	Neuropsychological functions	WAIS-R WSCT WAB (*Western Aphasia Battery*) Benton's Facial Recognition Line orientation Three dimensional construction tests Wechsler Memory Scale [Russell revision]

(Continued)

TABLE 1 | Continued

Article	Country	DM form	Sample	Investigation areas	Materials
Marchini et al. (67)	Italy	Not specified	24 DM1, 39 healty controls	Cognitive functions and other diseases related to DM	WAIS
Meola et al. (50)	Italy	Childhood, juvenile and Adult-onset form	19 DM2, 21 DM1	Cognitive functions and psychological characteristics	SCID (*Structured Clinical Interview*) MMSE Battery: TMT WCST TLT (*Tower of London Test*) ST (*Stroop test*) Lexical retrieval Computerized assessment of the multiple aspects of attentional performance
Modoni et al. (16)	Italy	Congenital and adult-onset forms	70 DM1 (10 congenital form, 60 Adult-onsetadult-onset)	Cognitive functions	Neuropsychological battery MMSE Subtest WAIS: RAVLT Rey-Osterrieth figure recall Digit Span Phonological and Semantic Word Fluency Raven Colored Progressive Matrices Temporal Rule Induction and SCWT TMT-B
Palmer et al. (68)	USA	All (except for congenital form)	21 DM1 (7 mMD and 14 pMD), 10 normal controls	Cognition and personality	MCMI (*Millon Clinical Multiaxial Inventory*) WAIS-R Wechsler Memory Scale RCFT Hooper Visual Organization Test Stroop A, B WSCT
Peric et al. (28)	Serbia	Childhood, juvenile, and adult-onset form	62 DM1	Personality	MIRS RSPM (*Raven's Standard Progressive Matrices*) MMCI SF-36 (*Short Form Health Survey*) [serbian version] INQoL
Perini et al. (69)	Italy	Adult-onset	17 DM1, 20 unrelated normal controls, 10 patients with SMA	Cognitive functions and psychiatric disorders	WAIS SADS (*Schedule for Affective Disorders and Schizophrenia*) MDRS (Muscular Disability Rating Scale) Event–Related Potentials
Prevost et al. (70)	Canada	All forms	308 subjects (44 carriers of DM1 gene and 264 non-carriers)	Psychological well-being after genetic test for DM	Questionnaire: Subjects were asked to complete items related to their social, economic and demographic background, the referring person to DNA testing, the reasons for testing and the recall of test result. PSI (*Psychiatric Symptom Index*)
Romeo et al. (71)	Italy	Not specified	50 DM1, 14 DM2, 44 healty subjects	Cognition and brain damage	MRI images MIRS, MRC scale Raven's progressive Matrices (PM47) Stroop (Word, Colour, Colour-Word) Fluency tests Wechsler Memory Scale CBT RCFT
Rubinsztein et al. (72)	UK	All except for congenital form	36 DM1	Memory and cognitive functions	Medical Research Council examination technique. NART (*National Adult Reading Test*) MMSE RBMT (*Rivermead Behavioural Memory Test*) WCST

(Continued)

TABLE 1 | Continued

Article	Country	DM form	Sample	Investigation areas	Materials
Sansone et al. (17)	Italy	Not specified	56 DM1, 29 DM2 (follow-up: 20 DM1, 13 DM2)	Cognitive decline in longitudinal study	MRC scale MMSE RPM (*Raven's Progressive Colored matrices*) Token test Digit Span forward and Spatial Span Story Recall and Rey Recall RCFT TMT-A and B, TEA (*Alertness and Divided Attention*) TLT
Sansone et al. (73)	Italy	Not specified (probably congenital excluded) "age- and disease duration-matched, moderately-affected, ambulatory patients"	66 Italian patients with SMC, skeletal muscle channelopathies; 422 DM (DM1: 382; and DM2: 40) as control group	Quality of Life in neuromuscular disorders	INQoL SF-36 MMSE
Serra et al. (40)	Italy	Childhood and adult-onset forms	27 DM1, 16 healty controls	Cognitive functions and psychological characteristics; f-MRI	MMPI-2 neuropsychological battery fMRI
Serra et al. (39)	Italy	All forms (in particular adult-onset form)	20 DM1, 18 healty controls	Cognitive functions and Theory of Mind	MMSE MIRS WAIS-R RMET (*Reading the Mind in the Eyes*) ToMstory tests fMRI
Sistiaga et al. (27)	Spain	All except congenital form	121 DM1, 54 control subjects	Cognition and personality	MCMI-II WAIS-III MIRS ESS
Steyaert et al. (74)	Holland -Belgium	Congenital or juvenile form	16 DM1	Psychological symptoms and cognitive functions	WISC-R or WAIS CBCL (*Children's Behavior Checklist*) Child Depression Scale or BDI (*Beck Depression Inventory*) ADIKA (*Diagnostic Interview for Children and Adolescents*) [parent's interview]
Turnpenny et al. (75)	UK	All forms	55 DM1, 31 healty controls at risk	Cognitive functions	WAIS [short version]
Van Spaendonck et al. (76)	Netherlands	Early adult and adult MD	26 DM, 25 control subjects	Cognition	MDRS (*Muscular Disability Rating Scale*) MRC Zung depression scale WAIS CVLT (*California Verbal Learning Test*) Stroop test TMT B-A VF WCST
Winblad et al. (77)	Sweden	Adult-onset form	46 DM1, 31 healthy controls, 37 in contrast group	Psychological characteristics	TCI (*Temperament and Character Inventory*) [Swedish version]
Winblad et al. (18)	Sweden	Adult form (not mild)	47 DM1	Cognitive functions	WAIS-R FAS (verbal fluency) RCFT (*Rey Complex Figure Test*) SCWT-B (*Stroop Color and Word Test B*) RAVLT (*Rey Auditory Verbal Learning Test*) WCST (*Wisconsin Card Sorting Test*) TMT-A TMT-B (*Trail Making Test A and B forms*)

(Continued)

TABLE 1 | Continued

Article	Country	DM form	Sample	Investigation areas	Materials
Winblad et al. (78)	Sweden	Adult-onset form	50 DM1, 41 healty controls	Psychological characteristics and facial emotion recognition	POFA (*Ekman and Friesen's Pictures of Facial Affect*) WAIS-R RAVLT RCFT COWAT (*Controlled Oral Word Test*); WCST CWT (Color Word Test) TMT TCI
Winblad et al. (21)	Sweden	Adult-onset form	31 DM1, 47 subjects in a clinical contrast group	Psychological symptoms (depression) and cognitive functions	BDI (*Beck Depression Inventory*) [Swedish version] MIRS tests: Vocabulary FAS Visual construction RCFT Block design Picture completion RAVLT TMT Digit symbol Digit span Spatial span SCWT WCST
Winblad et al. (23)	Sweden	Adult-omset form	37 DM1	Cognitive functions at follow-up	MIRS FAS Arithmetic Block design RCFT (*Rey Complex Figure Test*) RAVLT TMT-A, TMT-B Digit Span Spatial span SCWT-B WCST

15, 18, 22, 26, 27, 30, 35, 39, 51, 53, 56, 58, 61, 62, 66–69, 74, 76, 78).

For the assessment of the psychological characteristics, we adopted the SCL-90-R, based on the Canadian study of Bertrand et al. (26); since this test allows to inquire nine psychological dimensions and not only depression or anxiety that are used in many other studies (21, 52, 54, 57, 60).

The Clinical Psychology Service of the IRCCS Policlinico San Donato was activated in August 2016 and successively a collaboration with the Neurology Unit was established. A neuropsychological and psychological assessment research protocol on DM1 patients was reviewed and approved on the 24th of October by the ethical committee Ospedale San Raffaele in Milan, Italy (registration number 135/INT/2017) and was conducted according to the principles expressed in the Declaration of Helsinki, the institutional regulation and Italian laws and guidelines. Written informed consents were obtained from the patients.

Sample Characteristics

In our study, 31 DM1 patients referred to the Neuromuscular Center of the IRCCS Policlinico San Donato and registered in the Italian Registry for "Myotonic Dystrophy Type 1 and Type 2" were enrolled.

The following inclusion criteria were utilized: (i) age equal to or above 18 years; (ii) patients able to comprehend the conditions of the study and to participate to the entire duration of the study. Congenital and infantile form, pregnant patients or women who are breastfeeding and patients with IQ $\leq$ 91 were excluded. Only patients with IQ in the norm or above (IQ 92–109 normal/110–126 $>$ normal) were enrolled in order for them to be able to complete the whole neuropsychological and psychological assessment.

Patients were subdivided according to the five clinical forms based upon the age of onset as recently proposed by De Antonio et al. (6): (i) congenital form (onset from birth to 1 month); (ii) infantile form (onset from $>$1 month to 10 years); (iii) juvenile form (onset from 11 to 20 years); (iv) adult form (onset from 21 to 40); v) late onset form (onset after the age of 40 years).

Patients were categorized according to CTG expansion range: E1 (51–150), E2 (151–500), E3 (501–1,000), E4 (>1,000) (16).

Muscular impairment was assessed using: (i) Muscular Impairment Rate Scale (MIRS) between 1 and 5: 1 representing no muscular impairment and 5 representing severe muscular weakness; (ii) Medical Research Council Scale (MRC), a standard muscle power test, which grades on a scale of 0–5 in relation to the maximum expected for that muscle.

We measured individual daytime sleepiness with the Epworth Sleepiness Scale (ESS), a self-reported questionnaire that asks the patient to score (from zero to three) their likelihood of falling asleep in eight routine life situations. A final score ranging from 0 to 9 is considered normal; a score from 11 to 15 is typical of mild to moderate sleep apnea; a score of 16 and above is associated with severe sleep apnea.

Methods

The design of the study is evaluative, observational and prospective.

The assessment process was conducted by a neuropsychologist of the Clinical Psychology Service of the IRCCS Policlinico San Donato. The protocol administration lasted for an average of 90/110 min and included the following steps:

- Clinical and Neuropsychological Interview
- Neuropsychological assessment
- Psychological assessment

Clinical and Neuropsychological Interview

The purpose of the interview was to assess the following aspects of patients: personal history (educational, family, occupational, cognitive, social, medical, psychological), cognitive disorders, motivation and apathy, emotion and emotional disorders, self-control, sense of reality and psychotic type disorders, premorbid personality, personality modification, disease awareness, impact of disease on quality of life, family relationships and therapeutic alliance.

Global Cognitive Functioning Assessment

The Global Cognitive Functional Assessment was carried out through the administration of the Mini Mental State Examination (MMSE) (79).

This is a test for the assessment of intellectual and efficiency disorders and the presence of intellectual impairment. It consists of thirty items that refer to seven different cognitive areas: orientation in time, orientation in space, recording of words, attention and calculation, memory, language, constructive praxia. The total score is between a minimum of 0 and a maximum of 30 points. A score equal to or <18 indicates a serious impairment of cognitive skills; a score between 18 and 23 indicates a moderate to mild impairment, a score of 25 is considered borderline.

Executive Functioning Assessment

The Executive Functioning Assessment was carried out through the administration of the Frontal Assessment Battery (F.A.B.) (80).

It is a test for the assessment of the efficiency of executive functions and analyzes the following cognitive abilities: conceptualization, shifting/switching, planning, inhibitory control, sensitivity to interference, environmental autonomy (81). The cut-off limit for the adjusted score is set at 13.50.

Cognitive Domains Assessment Through ENB-2 Battery (Italian Version)

The ENB-2 assessment battery offers a qualitative and quantitative analysis of patient performance and is validated on the Italian population from 15 to 96 years and has the advantage of offering a shorter assessment time of cognitive domains.

The battery consists of 16 tests that analyze the following cognitive areas (82): short, long term and working memory, integration capacity, visual-spatial research, divided attention and attention shifting/switching, psychomotor speed, verbal comprehension, lexical recovery, logical and abstract reasoning, critical sense, capacity for discrimination, complex copy skill, praxis, and mental representation.

Psychological Evaluation Through Symptom Check List 90-R (SCL-90-R)

Similarily to Bertrand et al. (26), we selected the SCL-90-R questionnaire. It is a self-assessment questionnaire built to provide a standardized measure of an individual's current psychological and/or psychopathological status over to past week, applicable to normal or psychiatric populations of adults and adolescents (83). So far there is no evidence of other Italian studies on patients diagnosed with DM1 where a cross relation between Neuropsychological tests and SCL-90-R were performed. The objective in our study was to assess other psychopathological categories other than anxiety, depression, irritability and avoidant personality, and with the SCL-90-R there is also the possibility to take into consideration the 3 symptomatological indexes described hereunder. The nine primary dimensions investigated are: Somatization, Obsessive-Compulsive Dimension, Interpersonal Sensitivity, Depression, Anxiety, Hostility, Phobic anxiety, Paranoid ideation and Psychoticism.

The clinical information that can be obtained from the administration of SCL-90-R are also summarized into three indexes: Global Severity Index (GSI), Index of Disorder of Positive Symptoms (PST) and the Total Index of Positive Symptoms (PSDI)

The study of the psychometric properties of the SCL-90-R has provided satisfactory results for what concerns the internal coherence of the instrument and its reliability test–retest. Further studies confirmed the validity of construct and the predictive validity (Italian adaptation by E. Preti, A. Prunas, I. Sarno and F. Madeddu) (84).

Mean (and SDs) or median (and 1st and 3rd quartiles) as more appropriated, were used to describe continuous variables, while counts and percentage were used for categorical variables. The normality assumption was checked with the use of the Shapiro–Wilk test. Continuous parameter of diseases duration were analyzed using the two-sample Wilcoxon test for non-normally distributed data. Significance level was set at $P < 0.05$.

The SAS software, version 9.4 (SAS Institute, Inc., Cary, NC) was used for the analysis.

RESULTS

Sociodemographic, clinical, and psychological characteristics of DM1 patients are reported in **Table 2**.

A total of 31 patients with DM1 (19 men and 12 women) participated in this study. All patients were Italian and 77.4% were from northern Italy. Patients were classified as having juvenile (58.1%), adult-onset form (29.0%) and late onset (12.9%) forms of the disease.

The global cognitive functions screened through the MMSE were normal in the 96.8% of the entire DM1, only one patient reported a severe impairment in the spatial and temporal orientation, memory, language, praxis, attention and calculation.

Concerning the different specific cognitive domains, assessed through the ENB-2, it was shown that 80.6% of patients were in the range of normality and 19.4% were below the

TABLE 2 | Sociodemographic, clinical, and psychological characteristics of DM1 patients.

	All DM1 ($n = 31$)	Juvenile DM1 ($n = 18$)	Adult-onset DM1 ($n = 9$)	Late-onset DM1 ($n = 4$)
Age (years)	37.0 (25.0–50.0)	29.5 (24.0–40.0)	39.0 (36.0–52.0)	55.0 (48.0–58.5)
Gender (% Male)	61	55.6	55.6	100
Age at onset (*n*)	20.0 (16.0–25.0)*	16.0 (12.0–20.0)	31.0 (25.0–33.0)	43.0 (41.0–45.0)*
Disease duration (years)	14.0 (6.0–24.0)	15.0 (6.0–24.0)	14.0 (5.0–18.0)	8.0 (2.0–14.0)
CTG REPEAT RANGE (*n*)				
E1 (51–150)	2 (6.5%)	0	0	2 (50.0%)
E2 (151–500)	16 (51.6%)	10 (55.6%)	5 (55.6%)	1 (25.0%)
E3 (501–800)	6 (19.4%)	5 (27.8%)	1 (11.1%)	0
E4 (>800)	3 (9.7%)	2 (11.1%)	1 (11.1%)	0
not determined	4 (12.9%)	1 (5.6%)	2 (22.2%)	1 (25.0%)
MIRS	3.0 (2.0–4.0)	3.0 (2.0–4.0)	4.0 (3.0–4.0)	2.0 (1.0–3.5)
MRC	125.0 (112.0–127.7)	125.0 (111.0–128.0)	122.7 (115.0–125.0)	126.0 (112.8–130.0)
Epworth Sleepiness Scale	6.0 (4.0–9.0)	7.0 (6.0–12.0)	6.0 (4.0–7.0)	5.5 (3.0–6.0)
Education (years)	13.0 (8.0–13.0)	13.0 (13.0–13.0)	13.0 (8.0–16.0)	8.0 (8.0–8.0)
% employed	71.0	72.2	66.7	75
% in a relationship	45.0	22.2	66.7	100
ITALIAN REGION				
North	24 (77.4%)	12 (66.7%)	8 (88.9%)	4 (100%)
Center	3 (9.7%)	3 (16.7%)	0	0
South	4 (12.9%)	3 (16.7%)	1 (11.1%)	0
MMSE				
% in the norm range	96.8	94.4	100	100
ENB-2				
% in the norm range	80.6	72.2	88.9	100
FAB				
% in the norm range	71.0	61.1	77.8	100
% upper limits of the norm	16.1	22.2	11.1	0
% lower limits of the norm	9.7	16.7	0	0
% deficit	3.2	0	11.1	0
SCL-90-R				
% T-scores in the normal range				
Global severity index	80.6	83.3	77.8	75.0
Positive symptom total	87.1	88.9	77.8	100
Positive symptom distress index	83.9	88.9	77.8	75.0
Somatization	83.9	83.3	77.8	100
Obsessive-compulsive	90.3	94.4	77.8	100
Interpersonal sensitivity	80.6	83.3	77.8	75.0
Depression	80.6	88.9	66.7	75.0
Anxiety	96.8	100	88.9	100
Hostility	77.4	83.3	55.6	100
Phobic anxiety	90.3	94.4	77.8	100
Paranoid ideation	83.9	83.3	77.8	100
Psychoticism	83.9	88.9	77.8	75.0

Patients excluded since asymptomatic. Median value (interquartile range).

norm. The most negatively affected domains were: attention (25.8%), mental representation (29.0%), praxis (32.3%) and discrimination (22.6%).

The assessment of executive functions through the Frontal Assessment Battery (FAB) showed that 77.8% of patients had a normal frontal functioning and 22.2% had an impairment.

About one fifth of our DM1 patients showed SCL-90-R scores in the range of "normal" and the same distribution also occurred among the different DM1 forms.

19.4% of patients showed a moderate-high level of symptoms intensity index (GSI), 12.9% reported a moderate number of symptoms (PST) and 16.1% reported a moderate-high intensity level of the perceived symptoms (PSDI).

In relation to the nine symptomatic dimensions of SCL-90-R, 16.1% of patients refer moderate-high level of somatization (SOM), this also emerged during the clinical interview where patients often reported a strong feeling of uneasiness with their own body. Three patients (9.7%) showed obsessive-compulsive symptoms (O-C), others (9.7%) reported phobic anxiety (PHOB) and only one patient suffered from anxiety (ANX). 19.4% of the sample had a depressive state (DEP), this percentage is higher in comparison to the prevalence of depression in the normal population that stands around 2–5% (85). 19.4% of patients reported a high level of interpersonal sensitivity (IS), a typical psychological trait of people who experience feelings of inadequacy and inferiority with respect to others. Furthermore, the presence of hostility feelings (HOS in the 22.6%) and paranoid ideation (PAR in the 16.1%) can be seen as an indicator of existing relational problems. 16.1% of the sample reported the presence of psychotic symptoms (PSY) like social isolation and negative symptoms of schizophrenia.

It has been identified that the disease duration has a clinical impact on the cognitive performances assessed through the ENB battery. Patients who obtained results in the normal range of the ENB have a disease duration median of 6 years (5.0–22.0) vs. 23.5 (18.0–34.0) for those who obtained results below the norm ($P = 0.03$).

No statistical significance was found for other sample characteristics, such as % in a relationship, % employed and Italian region in correlation with ENB (respectively $P = 0.66$; $P = 0.18$; $P = 0.11$).

This might be due to the small sample size.

Illustrative Case Study

In this paragraph we illustrate a clinical case report of a family of four people (three diagnosed DM1 and one not affected by disease): Father, 53 years old, DM1 late onset form; Mother, 50 years old, not affected; Son 1, 25 years old, DM1 juvenile form; Son 2, 21 years old, DM1 juvenile form (neuropsychological and psychological data collected during the first visit are available in **Table 3**).

This family was selected in order to test a family-centered psychosocial care program since the family members have always been involved in the Italian Myotonic Foundation activities, as well as actively promoting initiatives around the DM1 disease.

Since this family is representing most of the disease forms, it might be representative of the various ages at disease onset and the different neuropsychological and psychological functioning as well as providing examples of different coping capabilities.

We designed a family-centered psychosocial care program in order to support the well-being of the different members. The main needs were different and were inferred from the SCL-90-R results and what emerged from the first clinical interview. The main objective of family-centered psychosocial care program was to strengthen the emotional resilience of chronically ill patients and their families. It aims to enhance a "holistic" treatment of the patient and the family as "a whole"—that is, from a body and mind perspective—and the psychosocial development of the patient and his or her family (86).

We met the father twice in order to assess his needs for support based on the fact that his two sons have also been diagnosed DM1. It immediately appeared that he had a good awareness of the disease and its implications as well as a strong resiliency. This also can be seen in **Table 3**, where all psychological dimensions are in the range of absence or normal. His demand for support was about taking care of his two sons and his wife who was feeling guilty because she was the only one not affected by the disease.

Son 1 is the most serious case in the family. From the very first clinical interview a clear picture of psychological pain and isolation from any social relationship emerged. The patient had just been left by his girlfriend since she was unable to cope with his disease and did not see any possible future in their relationship. He participated to 4 monthly psychotherapy sessions, in which the topics were the elaboration of the breakup and disease acceptance. The patient had no cognitive impairment but scored highly on the following psychopathological traits; O-C, DEP, PAR, PSY with also a high Global Severity index.

Son 2 demonstrated a high intelligence, with very quick reaction times and a very positive outlook on life. He only reported that he felt a bit excluded in the family since everyone was focused on Son 1 for his serious health conditions. He participated to five psychological sessions and during the clinical interviews it emerged that he was basically neglecting the fact the he was diagnosed DM1. There was a focus on the creation of awareness by using his strong self-perception as a solid point to be exploited. In fact, he achieved the best results in the cognitive performance and did not show any psychopathological traits.

The mother reported a strong sense of guilt for being healthy and a strong fear of losing her children. In order to protect them she was not allowing herself to feel any emotion since she wanted to appear strong and protect them from everything. In the psychological sessions she was allowed to express her feelings and to work on the acceptance of the situation.

The following indications were given at an individual level when it comes to the family dynamics:

- Openly sharing information, emotions and fears In order to reduce the stress and anxiety level related to DM1, it was important to provide a clear disease perspective to the patients and family members in collaboration with the neurologist. By doing so there was also a verification of the disease awareness among the different family members through the psychotherapy sessions;
- Being realistic and coping with what the daily life was presenting them. It was important to work on a program to

TABLE 3 | A neuropsychological and psychological assessment of a family.

	Cognitive functions			Psychological dimensions (SCL-90-R)*											
	MMSE	ENB-2	FAB	GSI	PST	PSDI	SOM	O-C	I-S	DEP	ANX	HOS	PHOB	PAR	PSY
Father	30/30	86	18/18	45	46	45	40	48	40	46	51	46	45	44	46
Son 1	30/30	85	18/18	76	58	88	56	84	64	75	56	60	43	84	85
Son 2	30/30	90	18/18	45	45	47	56	45	42	44	42	48	48	46	42

T-score : 25–55 (absence/normal), 55–65 (moderate), 65 > (high).

enhance a normal flow of life and step out from a pathological mode, by living in the here and now;
- Respecting the weaknesses of each other in order to provide mutual support;
- Psychological support once a month to monitor the situation by offering a dedicated individual session in order to enable the patients and their family members to share their fears, emotions and concerns.

Table 3 indicates the neuropsychological and psychological results of the family where three members have been diagnosed with DM1.

The data shown in **Table 3** are referring to the neuropsychological and psychological dimensions assessed during the first visit of the family members. A follow up assessment will take place at the end of the psychotherapy sessions by the end of 2018.

From a clinical observation perspective, we noticed an improvement of the general mood of son 1 who has developed a social network, has started socializing and has also started a part-time job showing an increased awareness and new abilities to cope with his disease. The improvement of the clinical symptoms of son 1 is having positive effects on the whole family with reduced stress for all members and a more supportive family environment can now be observed. Follow up data will be available at the end of the year.

DISCUSSIONS AND CONCLUSIONS

Recently in our hospital, the Clinical Psychology Unit of the IRCCS Policlinico San Donato in collaboration with the Neurology Unit has developed a battery of tests to evaluate cognitive assessment, personality traits and depression based on the results obtained by the Canadian team (26). A small group of DM1 patients has been tested with the intent to give them the possibility to access to psychological support other than to standard clinical care.

Regarding the main objective of this study, our results show relevant personality impairment in DM1 patients, mainly hostility, depression and interpersonal sensitivity. These symptomatic dimensions could be also explained with low empathy and low collaboration that Winblad measured with the Temperament and Character Inventory (TCI) (87).

The question which still remains open in our cohort is if these psychopathological traits were evident before or after the DM1 was diagnosed (88).

In our sample, cognitive impaired DM1 patients have a general tendency to maximize or minimize their positive symptoms and as shown in the literature, our DM1 patients tend to have a deficit in relations due to a low social cognition functioning (ToM, Theory of Mind) (38).

When it comes to the neuropsychological aspects, we have noticed that most negatively affected cognitive domains were praxis, mental representation, attention and discrimination.

Both neuropsychological and psychological impairments might have an impact on the patients' perception of quality of life. In fact, both quality of life and the patients' actual health, can be influenced by two main factors; decisions made by physicians and the patient's compliance. When a health condition is particularly serious, decisions regarding a diagnosis or treatment can often make a difference between life and death. For this reason, it is relevant for physicians to be able to make the best possible decisions and that the patients can understand what their clinical situation is and to follow the medical recommendations given (46).

In line with with our additional objective the emerging clinical implications and available data can be used as a basis to provide neurocognitive rehabilitation programs as well as psychological support program to DM1 patients and their families. Our study shows a bigger percentage of cognitive impairment when it comes to the praxis, mental representation, attention and discrimination domains, for which we are designing group and individual rehabilitation programs focused at the improvement of these functioning. The program will be available as soon as the research is completed and a bigger sample is available.

Since the study started last November 2017 and due to the small current sample size, we decided to describe the population without other inferential statistical analysis, which will be integrated in a later phase of the study when more data will be available.

At the moment we cannot envisage any effective medical treatment for DM1 patients and the data available in this research could effectively be used to work on DM1 patients' empowerment by making use of their cognitive reserve and stimulate through proper neuro-rehabilitation programs the main affected cognitive domains and offer psychological support program. The rehabilitation protocol will focus on attention, memory, praxis executive functions while on the psychological side enhance the disease awareness and patients' coping strategies.

Our transversal study confirms the results of the longitudinal trial of Winblad et al. (23), who highlighted a cognitive

deterioration at follow up time hence confirming the relation between the disease duration and cognitive performances.

This confirms the core importance of designing an effective rehabilitation protocol at the end of the data collection.

In these days, patients spontaneously search for information on the internet, and this might help them get accurate information but it may also lead them to getting lost on the web, getting information from unknown sources and inaccurate information. In order to get an effective involvement of patients in their care and their increased engagement, it is essential to enact strategies to improve their health literacy (45). It has been observed in the medical settings that usually patients require more information than they are given from health professionals, and they feel that they would like to be more involved in therapeutic procedure (44).

This is very much aligned to the innovative P5 approach, which represents the psycho-cognitive aspects to be considered in order to empower the patient, increase quality of life and transform them from a passive recipient into an active participants in the treatment process (46).

Because of the inherited nature of DM1, a diagnosis in one individual has implications for other family members, hence the care system is addressed to the family as a whole. Although the goal is clear, there are many challenges to proceed in this direction and it is necessary to create occasions where the patients have the possibility to be empowered. This means putting the patients in a condition that they feel they have control on their disease which in turn leads to an improvement in therapeutic outcomes (45).

In conclusion, in line with previous studies, there are indications that also the Italian patients have a high probability of developing psychiatric disorders. More data are necessary in order to make inferences about the relationships between psychosocial, neuropsychological and medical variables. Studies about the efficacy of interventions specifically established for these patients and their family members are necessary.

The current limitation is the effect of the small sample size. The complete analysis with the evaluation of primary and secondary end points of this study will be available when data collection is finished.

AUTHOR CONTRIBUTIONS

EC proposed the study design and the idea, analyzed the literature, its development and gave the final approval of the manuscript. EGB evaluated the neuropsychological and psychological assessment of patients, contributed to the development and the revision of the work and, agreed to the approval of the manuscript. MB executed the statistical analysis and contributed to the development and revision of the work and, the agreed to the approval of the manuscript. RC executed the histopathological and the molecular biology analysis and, contributed to the development and revision of the work and, agreed to the approval of the manuscript. BF provided the clinical data and contributed to the development and revision of the work and, agreed to the approval of the manuscript. EB provided the clinical data and contributed to the development and revision of the work and, agreed to the approval of the paper. GM organized the meeting, contacted the patients and supervised the medical aspects of the patients. GB also contributed to the development and the revision of the work and agreed to the approval of the manuscript. SB made substantial contributions to the analysis and the interpretation of the data in the manuscript and, revised it substantially. SB approved the final submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2018.00751/full#supplementary-material

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frontiers in Neurology

REVIEW
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Current Progress in CNS Imaging of Myotonic Dystrophy

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Neuroimaging in myotonic dystrophies provided a major contribution to the insight into brain involvement which is highly prevalent in these multisystemic disorders. Particular in Myotonic Dystrophy Type 1, conventional MRI first revealed hyperintense white matter lesions, predominantly localized in the anterior temporal lobe. Brain atrophy and ventricle enlargement were additional early findings already described almost 30 years ago. Since then, more advanced and sophisticated imaging methods have been applied in Myotonic Dystrophy Types 1 and 2. Involvement of actually normal appearing white matter and widespread cortical affection in PET studies were key results toward the recognition of diffuse and not only focally localized brain pathology *in vivo*. Later, structural abnormalities of both, gray and white matter, have been found in both forms of the disorder, albeit more prominent in myotonic dystrophy type 1. In Type 1, a consistent widespread cortical and subcortical involvement of gray and white matter affecting all lobes, brainstem and cerebellum was observed. Spectroscopy studies gave additional evidence of neuronal and glial damage in both types. Central questions regarding the origin and spatiotemporal evolution of the CNS involvement and its relevance for clinical symptoms had already been raised 30 years ago, however are still not answered. Results of correlation analyses between neuroimaging and clinical parameters are diverse and with few exceptions not well reproducible across studies. It may be related to the fact that most of the reported studies included only small numbers of subjects, sometimes even not separating Myotonic Dystrophy Type 1 from Type 2. But this heterogeneity may also support the current point of view that the clinical impairments are not simply linked to specific and regionally circumscribed structural or functional brain alterations. It seems more convincing that disturbed networks build the functional and structural substrate of clinical symptoms in these disorders as it is proposed in other neuropsychiatric diseases. Consecutively, structural and functional network analyses may provide additional information regarding the link between brain pathology and clinical symptoms. Up to now, only cross-sectional neuroimaging studies have been published. To analyze the temporal evolution of brain affection, longitudinal studies are urgently needed, and systematic natural history data would be useful to identify potential biomarkers for therapeutic studies.

Keywords: myotonic dystrophy, neuroimaging, MRI, PET, VBM, DTI, fMRI, brain

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INTRODUCTION

Myotonic Dystrophy Type 1 (DM1) and Type 2 (DM2) are autosomal dominantly inherited multisystem disorders with overlapping clinical phenotypes. Structural and functional brain involvement is highly prevalent and clinically relevant in both disorders.

Various neuroradiological techniques have been applied over the years to examine the morphological structure as well as the function of the brain in DM1 and DM2. Methods range from conventional cranial computed tomography (CT) that were used in early days of brain imaging to advanced magnetic resonance imaging (MRI) techniques like functional MRI (fMRI). Various morphological MRI techniques have been applied to examine brain structure in myotonic dystrophies including conventional morphological MRI sequences, volumetric MRI methods (normalized brain and cortical volumes, respectively), brain parenchymal fraction (BPF), surface-based morphometry techniques (SBM) to analyze callosal body volumes, voxel-based morphometry (VBM) to analyze gray matter (GM), and white matter (WM) volumes/densities using voxel-based approaches, and diffusion MRI techniques (DTI) to examine WM microstructural integrity by voxel- or ROI-based approaches. Fluorodeoxyglucose positron emission tomography (FDG-PET) has been used to investigate the cerebral glucose metabolism in myotonic dystrophies. To analyze cerebral perfusion, ^{99m}Tc-ECD and HMPAO single-photon emission computed tomography (SPECT) as well as H_2O^{15}-PET were applied. Analyses of cellular and neuronal markers have been performed by Proton-MR-spectroscopy (^{1}H-MRS). Functional neuroimaging techniques were introduced to gain more insight into brain functioning and cerebral networks in myotonic dystrophies. Thus, functional MRI (fMRI) using e.g., motor tasks and resting-state fMRI have been applied in DM1 patients. Recently, transcranial B-mode sonography was used in adult-onset DM1 and DM2 patients for the first time to evaluate the echogenicity of brainstem and basal ganglia as well as ventricle diameters.

This review is based on studies reporting neuroimaging results in DM1 or DM2 patients applying at least one of the following methods: (functional) MRI, magnet resonance spectroscopy (MRS), SPECT, PET, or ultrasound. Search was done in Pubmed-database until March 1 2018, studies should be written in English and the full text of the study available. We also performed cross-referencing to identify articles potentially missed by our search.

Abbreviations: AD, axial diffusivity; BPF, brain parenchymal fraction; CBF, cerebral blood flow; Cho, choline; Cr, creatine; CTG, CTG repeat length in DM1 patients; DM1, myotonic dystrophy type 1; DM2, myotonic dystrophy type 2; DMN = default mode network; DTI, diffusion tensor imaging; FA, fractional anisotropy; GM, gray matter; MD = mean diffusivity; MIRS, muscular impairment rating scale; MR, Magnetic resonance; MRS, magnetic resonance spectroscopy; NAA, N-acetyl aspartate; NAWM, Normal appearing white matter; PET, positron emission tomography; RD, radial diffusivity; ROI, region of interest; SPECT, single photon emission computed tomography; VBM, voxel-based morphometry; WM, white matter; WMHL, white matter hyperintense lesions.

Myotonic Dystrophy Type 1

First cranial CT scans in DM1 had been conducted and published in the 80s and were the first real proof of a morphological brain affection in this disorder. These early neuroimaging data had given rise to a couple of scientific comments on clinical and radiological observations in DM1 that were published subsequently (1–3). DM1 patients showed increased ventricular surface areas and asymptomatic areas of focal cerebral atrophy (4). Further cranial CT studies gave evidence of microcephaly and thickening of the calvarium thus confirming cranial hyperostosis as a possible sign of brain pathology in DM1. Some patients also presented with basal ganglia calcification (5).

To our knowledge, the first publication on brain MRI in DM1 also included the first published cerebral CT image of one DM1 patient. The MRI study made use of a 0.5 T scanner for brain imaging. There was an increased incidence of ventriculomegaly in DM1 patients. Furthermore, this study gave first evidence of periventricular hyperintensities in DM1 patients when compared to controls (6).

First neuroimaging data in myotonic dystrophies included cranial CT of the brain and skull as well as 0.5 T brain MRI in congenital DM1, juvenile- as well as adult-onset patients and had been restricted to the type 1 form of the disease. In contrast, the first brain imaging study in DM2 was published only in 1997 (7). In general, CT imaging in those early days showed ventricular enlargement particularly in the congenital form of DM1, microcephaly, thickening of the calvarium, diffuse brain atrophy, cortical atrophy, and white matter hypodensities. Early reports on conventional morphological brain MRI findings in DM1 describe a wide variety of abnormalities ranging from dilated Virchow-Robin spaces, WM hyperintense lesions (WMHL) to cerebral atrophy(6, 8–12).

In the following years, several conventional structural brain MR studies were conducted in DM1 using MR scanners with field intensities of up to 1.5 T. The first "high-field" 3.0 T brain MRI studies however were performed in 2011 in congenital and juvenile-onset DM1 (13) and in adult-onset DM1 and DM2 patients (14). The latter study of Minnerop et al. included conventional structural brain MRI whereas the high-field study on congenital and juvenile-onset DM1 of Wodzniak et al. was focused on DTI techniques.

Conventional Morphological Brain MRI in DM1

Data base research for original articles published from 1988 to 2018 identified 40 systematic neuroimaging studies that included conventional structural/morphological MRI techniques in various series of DM1 patients using field intensities of 0.5–3.0 T (1, 6, 9–11, 14–46).

The numbers of studies using conventional, non-quantitative structural MRI techniques decreased in the recent years due to the implementation of more advanced and quantitative techniques like VBM and DTI as well as fMRI technologies.

The number of investigated patients by conventional MRI ranged from 2 to 60 patients per study, not respecting a review article on 66 DM1 patients and a very recent thorough review article on brain imaging in DM1 (23, 47). Most examinations focused on the classical adult-onset form of DM1, whereas few

patient series included small numbers of congenital DM1 patients and juvenile-onset forms (6, 18, 21, 23, 26, 35).

Brain MR images were usually evaluated according to WM and GM structural abnormalities. Most studies were merely descriptive, especially in the beginning of MRI application in DM1, and not all studies compared patient data against healthy control subjects. Later, there have been strong attempts to quantify particularly the data on WM changes, and several scores to describe and quantify WMHL have been applied. WM lesions scores used in DM1 imaging studies included e.g., the age-related white-matter changes scale [ARWMC, (48)], and later also a visual scale according to a modified version of the Fazekas scale (43, 49). Due to the availability of WMHL scales and scores, the most uniform and comparable data of conventional morphological brain MRI are available for WMHL in DM1. In contrast, methods of GM evaluation are much more diverse and highly rater-dependent which might contribute to more controversial results on GM findings in DM1 conventional brain MRI studies. More advanced MRI techniques like VBM have largely replaced conventional brain MRI in DM1 to analyze GM, which allows a more rater-independent and quantitative analysis of GM abnormalities like cerebral atrophy in DM1.

White Matter Findings in DM1 Using Conventional Morphological Brain MRI

In general, WM changes and WMHL have frequently been reported in patients with DM1 (14, 24, 31). Also, thinning or atrophy of the callosal body has been reported most frequently in congenital DM1 but also in adult-onset forms of the disease (18, 19, 21, 46). WMHL in DM1 are predominantly located in frontal and temporal lobes (**Figure 1A**). Particularly, anterior temporal WML (ATWML) show a high prevalence in DM1 patients (**Figure 1B**). ATWML and a temporopolar WM pathology were identified in various and independent DM1 cohorts and constitute a highly robust finding over more than two decades of brain imaging. ATWML have been described early in DM1 (8) and could be reproduced only recently in brain MRI studies using more elaborated scanning techniques (43, 45, 46). ATWML seem to be a rather specific presentation of DM1 especially when compared to DM2 where this characteristic brain affection is usually not present (7–11, 14, 24, 31, 38).

The natural history of WM affection in DM1 is still a matter of debate and even conventional structural brain MRI data are controversial in this respect. Most cross-sectional analyses investigating the presence and extent of WMHL gave evidence of a potential progress over time or more pronounced WMHL in older patients when compared to children or adolescents with DM1, with age or disease duration used for correlation analyses. However, others did not find significant correlations with age or a significant increase of WML with disease progression (9, 11, 20, 23, 45, 46, 50, 51). In summary, longitudinal data on the presence and extent of WM abnormalities and WML in DM1 are scarce, and a systematic analysis of WM affection over time in DM1 patients compared against healthy controls by conventional MRI is still missing.

Gray Matter Findings in DM1 Using Conventional Morphological Brain MRI

In a recent review article, brain imaging studies on DM1 using magnetic resonance spectroscopy (MRS), fMRI, CT, ultrasound, PET, SPECT, but also conventional brain MRI has been analyzed. The authors had extracted data from a total of 81 studies on patients with DM1 (Embase, index period 1974–2016 and MEDLINE, index period 1946–2016). In conclusion, general brain atrophy and widespread GM volume reductions were reported in all cortical lobes, the basal ganglia, and cerebellum in DM1 (47).

Brain atrophy was described as a characteristic finding in DM1 very early. GM abnormalities reported in the literature by use of conventional morphological brain MRI techniques include ventricular enlargement, diffuse cortical atrophy, global GM reduction, focal brain atrophy in various cerebral lobes, the hippocampus, and basal ganglia. Cortical and subcortical GM atrophy is reported to be mostly symmetric and seems to be more pronounced in adult-onset compared to juvenile or congenital forms of DM1. Progressive GM loss in DM1 had been assumed earlier according to cross-sectional neuroimaging study results (14, 33, 38, 40, 44). However, there is discrepancy in the literature about the effect of including congenital DM1 patients on the extent of GM changes in neuroimaging analyses (38, 44). Some data hint to a more pronounced GM atrophy when congenital forms are included, others suggest the opposite. As in WM changes, the natural history of GM changes in DM1 is largely unclear, and longitudinal data on GM abnormalities are widely missing. To answer these still unsolved questions, systematic longitudinal brain imaging studies on large cohorts of congenital, juvenile- and adult-onset DM1 patients over time and against healthy controls would be needed.

Structural Brain Imaging in DM1

The development of observer-independent neuroimaging techniques allowed quantifying brain atrophy, and specific properties of the brain could be accessed and analyzed by the use of specific MR sequences.

Quantification of Global Brain Volume in DM1

Please see **Table 1** for technical details of the included studies. In 2003, Kassubek et al. estimated brain atrophy in DM1 via brain parenchymal fraction (BPF) (52). This method comprises the segmentation of T1-weighted MR sequences into GM, WM and cerebrospinal fluid and the calculation of BPF by dividing the sum of GM and WM fractions by the sum of GM, WM and cerebrospinal fluid fractions - so far representing the total intracranial brain volume. Since BPF decreases during healthy aging and females have higher BPF values (53), age- and sex-matched control groups are required. In DM1, BPF values were markedly reduced, but did not correlate with clinical parameters like disease duration, motor score, educational level, or CTG repeat length.

Antonini et al. compared GM and WM volumes (not fractions) between non-congenital DM1 patients and controls and found significant reduced volumes in DM1 patients (54). The negative correlation of age with GM volume was stronger

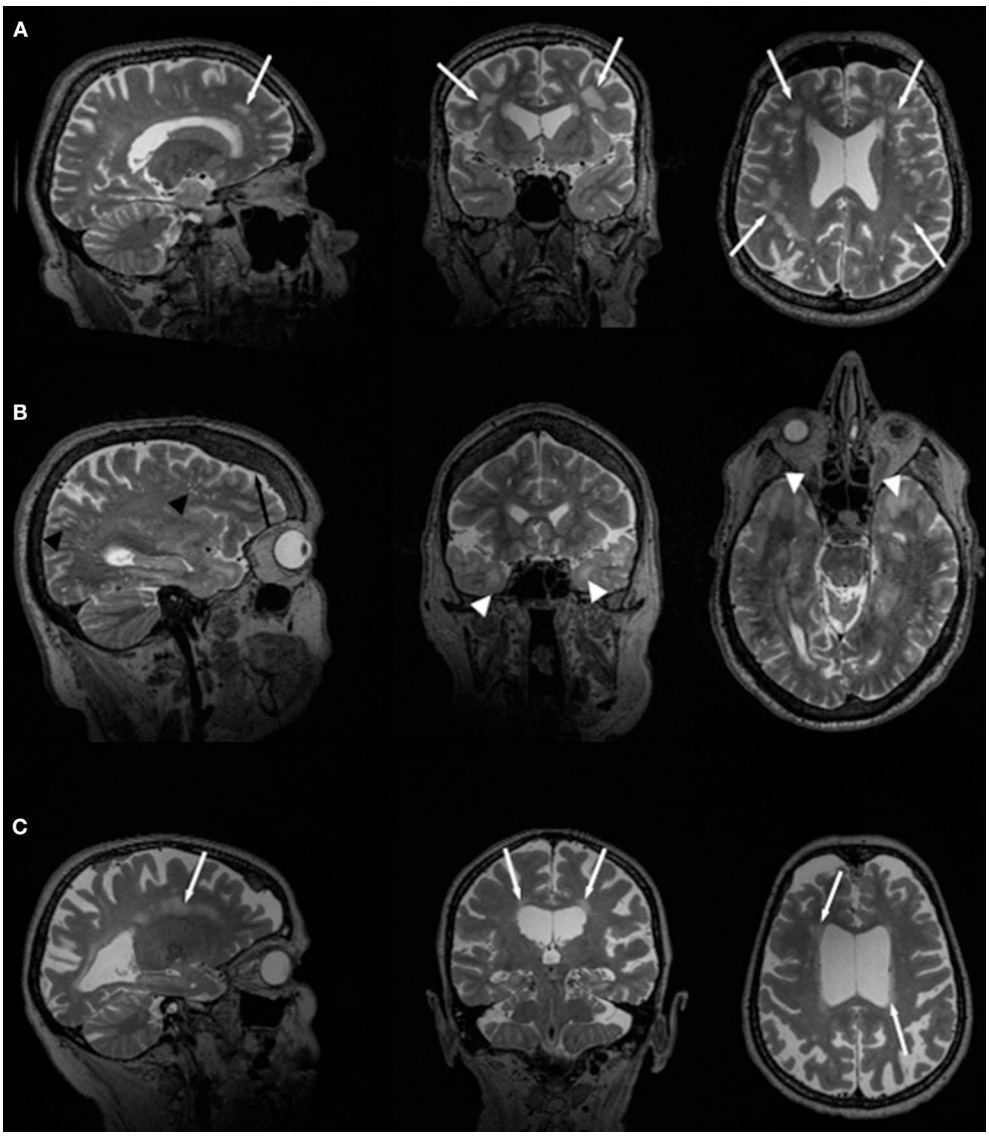

FIGURE 1 | Slices of T2-weighted MR images in two different DM1 **(A, B)** and one DM2 **(C)** patients. Global atrophy with ventricle enlargement is seen in all cases. White arrows indicate WMHL, white arrow heads point toward ATWML. Black arrow indicates frontal hyperostosis in DM1 patients, black arrow heads point toward dilated Virchow-Robin spaces.

in patients than in controls, while WM volume did not correlate with age—neither in patients nor in controls. Similar to Kassubek et al. they found no correlation between brain tissue volumes and clinical parameters {disease duration, muscular impaired rating scale (MIRS, (55)), CTG repeat length, or WMHL load}. Antonini et al. stated correctly that their cross-sectional design did not allow differentiation between neurodevelopmental volume loss, acquired atrophy or progressive neurodegeneration, but postulated a neurodevelopmental GM loss which progresses with ageing because of the more pronounced negative correlation with age. The lack of any correlation between GM volume and WMHL-load led to the suggestion that WMHL and cortical atrophy progress as two independent processes.

Regarding BPF, Weber et al. confirmed previous findings from Kassubek et al. but described now a correlation with disease duration and WMHL load (33, 52).

Schneider-Gold et al. confirmed supratentorial brain atrophy in DM1, mainly driven by GM atrophy and accompanied by enlargement of all ventricular spaces, which also correlated negatively with each other (41). Supratentorial atrophy correlated also inverse with MIRS. However, the temporal horn index (temporal horn volume/lateral ventricle volume), supposed as an indirect and sensitive regional measure for (para-) hippocampal atrophy was only non-significantly elevated. No atrophy of the cerebellum or the upper cervical cord was detected.

TABLE 1 | Quantification of brain volume in DM1.

Study	Modality	Estimated parameters	Group size	Age (M ± SD or range) [years]	Disease duration (M ± SD or range) [years]	CTG repeat length (range)	Correlation parameters
Kassubek et al. (52)	1.5T (T1)	BPF	10	36 ± 14	17 ± 10	110–1300	Disease duration, motor score, educational level, CTG
Antonini et al. (54)	1.5T (T1, T2)	GM/WM volume, TIV WMHL-load, VBM (GM)	22 (no congenital)	20–55	2–47	96–1570	Age, disease duration, MIRS, CTG, WMHL-load
Ota et al. (56)	1.0T (T1)	VBM (GM), corpus callosum volume	11 (no congenital)	56.6 ± 8.6	28.5 ± 15.5	+	Age, age at onset, disease duration, CTG (+)
Weber et al. (33)	1.5T (T1, T2, FLAIR)	BPF, VBM (GM)	14 (no congenital/early-onset)	37.2±14.2*	16.0 ± 9.6*	100–1300*	Disease duration (+), NPT (+), WMHL-load (+)
Minnerop et al. (11)	3T (T1)	VBM (GM, WM)	22 (no congenital/childhood onset)	43.1 ± 12.6	13.2 ± 7.0	80–1100	-
Franc et al. (57)	3T (T1)	GM, ROI-based	10 (congenital +adult-onset)	24–34/30–43	-	+	FA (+)
Caso et al. (38)	1.5T (T1)	VBM (GM)	51 (17 juvenile)	42 ± 10	19.2 ± 8.5	177–1534	Age, disease duration, CTG, NPT, sleepiness
Schneider-Gold et al. (41)	3T (T1)	VBM (GM, WM), volumentry total GM/WM, cerebellum, brainstem, upper cervical cord, ventricle	12 (no congenital/childhood-onset)	45 ± 13	18 ± 7	75–720	NPT (+), depression (+), daytime sleepiness, MIRS (+)
Serra et al. (40)	3T (T1)	VBM (GM)	10	41.8 ± 9.6	-	54–2000	CTG (+)
Baldanzi et al. (43)	3T (T1, FLAIR)	BPF, VBM (GM)	30 (only adult-onset)	44.6 ± 12.4	16.5 ± 11.8	E1, E2	Age, disease duration, NPT (+)
Zanigni et al. (44)	1.5T (T1, FLAIR)	VBM (GM), CT	24	38.5 ± 11.8	16.2 ± 10.8	E1, E2, E3	NPT, CTG, clinical scale
Cabada et al. (46)	1.5T (T1)	ROI- based (sub-) cortical GM-volume corpus callosum	40 (adult-onset)	37.3	-	+	Age (+), sleepiness (+), NPT (+)
Sugiyama et al. (58)	3T (T1)	VBM (GM), graph theory	28 (4 childhood, 10 juvenile)	42.5 ± 11.2	22.0 ± 12.4	133–3000	CTG, disease duration, age at onset, MIRS

*All included studies investigated patients in comparison with controls. BPF, brain parenchymal fraction, CSF, cerebrospinal fluid; CT, cortical thickness; CTG, CTG repeat length; E1, 50–150 CTG repeats; E2, 150–1000 CTG repeats; E3, >1000 CTG repeats; GM, gray matter; MIRS, muscular impairment rating scale; NPT, neuropsychological tests; ROI, region of interest; TIV, total intracranial volume (GM+WM+CSF); VBM, voxel-based morphometry; WM, white matter; WMHL, white matter hyperintensity lesions; (+), positive or negative correlation was found; * data refer to whole DM1 study group, including patients that did not undergo MRI.*

Up to now, Baldanzi et al. analyzed the largest group of DM1 patients with respect to BPF and found a correlation with visuo-spatial and executive performance (43).

Quantification of Regional Brain Volume in DM1

Please see **Table 1** for technical details of the included studies.

Voxel-based morphometry (VBM) allows analyzing local volume changes within the brain and is based on an automated segmentation procedure of T1-weighted MR images into GM, WM and cerebrospinal fluid in combination with normalization to tissue-class specific templates. The procedures result in smoothed probability maps for each tissue class which can be compared between groups.

The first VBM study in non-congenital DM1 patients was performed by Antonini et al. demonstrating a widespread pattern of volume differences, located in frontal, parietal, temporal cortex bilaterally, and left superior occipital gyrus. Subcortical atrophy of the left caudate was detected as well (54). Subsequently published VBM studies in non-congenital DM1 patients largely confirmed a pattern of widespread cortical GM reduction affecting all lobes and frequently involving pre- and postcentral gyrus (14, 33, 38, 40, 41, 43, 44, 56, 58). Hippocampus atrophy was described by only few VBM studies (33, 38, 44, 58) and in one study even correlated with episodic memory (33). Subcortical changes are also part of the pattern observed in non-congenital DM1 patients and involve striatum, thalamus and cerebellum, albeit to a variable extent in different studies. Cabada et al. applied a methodological different, ROI-based approach to estimate GM volume and observed additionally subcortical reduced gray matter volume in the nucleus accumbens and ventral diencephalon (46).

Up to now, Zanigni et al. has performed the only analysis of cortical thickness in DM1 patients (44). Although VBM and cortical thickness analyses address both GM in T1-weighted MR images, there are methodological differences. VBM analysis provides a mixed measure of GM volume, including different structural properties, such as cortical thickness, surface area, and cortical folding (59). By combining both techniques, complementary information regarding brain structure can be obtained. Zanigni et al. found in line with the widespread GM involvement in VBM studies reduced thickness within lateral-occipital cortex bilaterally, right precentral and left superior-parietal, superior-temporal, and fusiform cortices (44).

Only a few studies analyzed WM changes with VBM in non-congenital DM1 patients (14, 41). Minnerop et al. observed WM changes encompassing the entire corpus callosum, fornices, cingulum bundle, and confluent WM reductions in every lobe (14). Subcortical WM changes were visible at pontine level, along middle cerebellar peduncles and within cerebellar WM. Schneider-Gold et al. described reduced WM volume in corpus callosum, thalamus, and WM adjacent to the pre- and post-central gyrus left-sided (41). Ota et al. and Cabada et al. confirmed with ROI-based approaches reduced volume of the corpus callosum (46, 56).

VBM studies comparing patient groups with different disease-onset are very limited. Franc et al. found reduced GM volumes only in adult-onset DM1 patients, but not in patients with congenital onset (57). Caso et al. compared juvenile and adult-onset DM1 patients and found in patients with juvenile form only small regions of bilateral cortical atrophy in the pre- and post-central gyri, SMA, orbitofrontal, dorsal frontal, and lateral temporal cortices, parietal regions, occipital cortices, left cingulate cortex, and right thalamus (38). Zanigni et al. observed after excluding patients with congenital/childhood-onset unchanged subcortical GM changes while the cortical GM reduction was less pronounced than for the entire group (44). Similar, restricting the cortical thickness analysis to patients without congenital/childhood-onset lead to more circumscribed altered cortical thickness, affecting only left fusiform, lingual, and inferior temporal gyri.

Seven studies investigated correlation of CTG repeat length with GM volume, but only two detected a correlation. Ota et al. found a negative correlation between CTG repeat length and GM volumes of the bilateral motor area and right prefrontal cortex (56). Serra et al. found significant correlations with GM volumes of cingulate gyri, orbitofrontal cortices, and frontal poles bilaterally and left pre-central gyrus (40). So if any correlations with CTG repeat length were detected at all, it was associated with motor-related and frontal areas.

Performing regression analysis, Schneider-Gold et al. revealed an association of pontine WM changes with depression score (41). Most neuropsychological parameters did not correlate with imaging parameters. Only flexibility of thinking correlated with GM volume within left medio-parietal cortex, belonging to the secondary visual cortex. Correlation analyses in the study of Baldanzi et al. revealed an association between delayed recall of verbal memory test and the volume of left postcentral, left middle, and inferior temporal gyri and left supramarginal gyrus (43).

Cabada et al. analyzed the volume of (sub-) cortical GM in a ROI-based approach (46). Compared with controls, there was an increase rate of cortex volume loss associated with age. Sleepiness was associated with volume loss in right pallidum and right ventral diencephalon. Visuospatial impairment was significantly correlated with ventricle enlargement and volume loss in part of the corpus callosum, bilateral cingulated isthmus, right lateral occipital, and pericalcarine cortex.

The most recent study by Sugiyama et al. applied for the first time graph theoretical analysis to investigate network metrics of a network between predefined brain regions based on GM in DM1 patients (58). In spite of pronounced GM volume reduction according to afore executed VBM analysis, measures of global and local network organization did not differ between DM1 patients and controls—probably due to compensatory mechanisms. The parameter betweenness centrality (BC) estimates the number of shortest paths that traverse a given node. High BC values imply that the respective node is a highly central "hub" for anatomical connections. Although the number of hubs was reduced in DM1 patients, there was an increased BC in left fusiform, superior temporal gyrus, superior frontal gyrus, and right precuneus, and a decreased BC in right caudate nucleus and putamen. The authors assumed that the increased BC in the left fusiform gyrus might be related to abnormalities of face perception in DM1 patients. The decreased BC in the striatum reflecting reduced structural connectivity may be correlated with schizotypal-paranoid traits, as it was discussed by Serra et al. for reduced functional connectivity in the striatum (36).

Quantification of White Matter Alterations in DM1

Please see **Table 2** for technical details of the included studies.

In the last two decades an increasing number of advanced MR methods were developed to specifically analyze structural changes within WM.

Di Constanzo et al. performed the currently only study in adult-onset DM1 patients applying T2 relaxometry (62). This method has been shown to be useful in quantifying signal changes on T2-weighted images, on which also in DM1 characteristic abnormalities are frequently observed. Relaxation times estimated in normal-appearing white matter (NAWM) and (sub-) cortical regions (striatum, thalamus) were prolonged in particular in WM. Total WM T2 values correlated with age and disease duration, while no correlations were found for specific WM regions or for GM. The detected changes within NAWM had not been reported before and pointed for the first time toward a more diffuse involvement of WM in DM1. The correlations with age and disease duration, but not with CTG repeat length were interpreted as possible signs of progressive changes in disease course.

Naka et al. used magnetization transfer imaging to analyze WM in DM1 patients (60). This method is based on the interaction between (myelin-) bound and free protons and is frequently applied in demyelinating disorders. Magnetization transfer is induced by an MR pulse saturating bound protons and differences of the signal intensity before and after inducing

TABLE 2 | Quantification of white matter alterations in DM1.

Study	Modality	Estimated parameters	Group size	Age (M ± SD) [years]	Disease duration (M ± SD) [years]	CTG repeat length (range)	Correlation parameters
Di Constanzo et al. (62)	0.5T (T2-Relaxometry)	T2-relaxation time, ROI-based (GM, WM)	20	37.6 ± 13.8	11.8 ± 8.4	96–2930	WMHL, VRS (+), age (+), aget at onset, disease duration (+), VBR, CTG, MIRS
Naka et al. (60)	1.5T (T1, T2, FLAIR, MTI)	MTR, ROI-based, (NAWM, WMHL)	14	41.5 ± 10.5	14.3 ± 6.9	-	Disease duration (+), age, age at onset
Fukuda et al. (25)	1.5T DTI (d6, b500)	ROI-based (FA, MD) (NAWM, WMHL)	19	43.9 ± 10.9	13.7 ± 9.0	-	Age, age at onset, disease duration
Ota et al. (56)	1.0T DTI (d12, b700)	ROI-based (FA, MD) tractography of corpus callosum	11 (no congenital)	56.6 ± 8.6	28.5 ± 15.5	+	Age, age at onset, disease duration, CTG
Wozniak et al. (13)	3T DTI (d30, b1000)	ROI-based (FA, MD, AD, RD)	8 (congenital + juvenile onset)	13.8 ± 2.3	-	200–1700	NPT (+)
Minnerop et al. (11)	3T DTI (d30, b1000)	DTI-TBSS (FA, MD, RD, AD)	22 (no congenital/childhood onset)	43.1 ± 12.6	13.2 ± 7.0	80–1100	Age (+), disease duration (+), depression (+), fatigue (+), NPT CTG (+), MIRS (+)
Franc et al. (57)	3T DTI (d12, b1000)	ROI-based (FA)	10 (congenital + adult-onset)	29.5/38.3	-	+	GM (+)
Wozniak et al. (61)	3T DTI (d30, b1000)	probabilistic tractography, ROI- based (FA, MD, RD, AD)	16 (congenital, childhood/juvenile onset)	13.9 ± 3.0	-	(+)	NPT (+)
Wozniak et al. (37)	3T DTI (d30, b1000)	probabilistic tractography, ROI- based (FA, MD)	45 (juvenile/adult-onset)	38.4 ± 6.6	-	75–800	NPT (+), MIRS (+), CTG (+), sleepiness (+)
Caso et al. (38)	1.5T DTI (d65, b1000)	DTI-TBSS (FA, MD, RD, AD)	51 (17 juvenile)	42 ± 10	19.2 ± 8.5	177–1534	Age, disease duration, CTG, NPT (+), sleepiness, WMHL-load
Serra et al. (40)	3T DTI (d61, b1000)	DTI-TBSS (FA)	10	41.8 ± 9.6	-	54–2000	CTG (+), NPT (+), MIRS (+)
Baldanzi et al. (43)	3T DTI (d25, b1000)	DTI-TBSS (FA, MD, RD, AD)	30 (adult-onset)	44.6 ± 12.4	16.5 ± 11.8	E1, E2	Age, disease duration, NPT (+)
Zanigni et al. (44)	1.5T DTI (d25, b900)	DTI-TBSS (FA, MD, RD, AD)	24	38.5 ± 11.8	16.2 ± 10.8	E1, E2, E3	NPT (+), CTG, clinical scale (+)
Cabada et al. (46)	1.5T DTI (d30, b1000)	DTI-TBSS (FA, MD, RD, AD)	40 (adult-onset)	37.3	-	+	Age, NPT (+), sleepiness, WMHL-load (+)

All included studies investigated patients in comparison with controls. AD, axial diffusivity; b, b- factor [s/mm^2]; CTG, CTG repeat length; d, number of directions of diffusion-encoding gradients; DTI, diffusion tensor imaging; E1, 50–150 CTG repeats; E2, 150–1000 CTG repeats; E3, >1000 CTG repeats; FA, fractional anisotropy; MD, mean diffusivity; MIRS, muscular impairment rating scale; MTI, magnetization transfer imaging; NAWM, Normal appearing white matter; NPT, neuropsychological tests; RD, radial diffusivity; ROI, region of interest; TBSS, tract-based spatial statistics; VBR, ventricle-to-brain ratio; VRS, Virchow-Robin-spaces; WMHL, white matter hyperintensive lesions; (+), positive or negative correlation was found.

Magnetization transfer are measured. The authors found, consistent with the relaxometry–study, reduced MT ratios in WHML more than in NAWM, and in both a correlation with disease duration was observed.

The first diffusion tensor imaging (DTI) study in DM1 patients was performed by Fukuda et al. (25). DTI evaluates the magnitude and directionality of the diffusion of water molecules. The biophysical background of diffusivity changes in WM is still not fully understood. Next to myelin changes other structural abnormalities (e.g., axonal membranes) may play role. Within WM, diffusion is usually anisotropic ("directed") due to the structural constraints of fiber tracts. Pathological disturbances of these microstructural barriers alter the diffusion behavior of water molecules, and reduce for example the anisotropy of diffusion within WM. This anisotropic diffusion can be quantified with the parameter fractional anisotropy (FA) in which higher values indicate more anisotropic, directed diffusion. Next to FA other diffusivity parameters exist: Like FA, the parameters radial and axial diffusivity (RD, AD) are derived from the same mathematical tensor model underlying DTI. RD and AD are thought to define the amount of diffusivity in the direction (axial) and perpendicular (radial) to the direction of fiber tracts. In this respect, radial diffusivity is thought to reflect alterations of myelin or axonal membranes while axial diffusivity may reflect pathological changes of the axon itself. Mean diffusivity (MD) measures the general diffusivity without paying attention to the direction of the diffusivity.

Fukuda et al. (25) compared similar to Naka et al. (60) and Di Constanzo et al. (62) method-specific measures within WMHL and NAWM applying a ROI-based approach. And again, they found lower FA and higher MD values in both, WMHL and NAWM, but more pronounced in WMHL. But in contrast to Di Constanzo et al. (62) and Naka et al., they did not find any correlation with disease duration or any other clinical parameter and assumed a non-progressive character of changes within NAWM (25, 60, 62).

Ota et al. showed diffusivity abnormalities within the corpus callosum for the first time in non-congenital DM1 patients and linked them to reduced cortical GM volume in different lobes (56). Not affected were only parietal lobes and the isthmus of the corpus callosum, both connected by fiber tracts. Furthermore, CTG repeat length correlated with cortical GM loss – but not with diffusivity parameters within the corpus callosum. The authors postulated Wallerian degeneration as underlying pathology of callosal diffusivity changes.

In our own study (Minnerop et al.) we confirmed changes of diffusivity within the corpus callosum in adult-onset DM1 patients (sparing only parts of the splenium), but found in a voxel-based approach extensive and wide-spread abnormalities throughout the entire brain (14). This involved association fibers, limbic system fiber tracts and projection fibers within internal and external capsules, including also changes of the corticospinal tract within the posterior limb of internal capsules and at brainstem level. Reduced FA was mainly accompanied by increased MD and RD, but increased AD was also present. Subsequent DTI studies confirmed this pattern of widespread diffusivity abnormalities (38, 40, 43, 44, 46).

Congenital and juvenile-onset DM1 patients were shown to have as well generalized changes of diffusivity parameters (reduced FA, higher MD, AD, and RD) and this was later also confirmed for children and adolescents with DM1 applying a tractography approach (13, 61). Franc et al. used the same set of large ROIs as in Wozniak et al. (13) to investigate congenital and adult-onset DM1 patients and found reduced FA values in both groups with lower values for the adult-onset DM1 group (57). Caso et al. included the up to now largest group of DM1 patients into a multimodal imaging study, allowing even to compare the juvenile with the adult-onset form (38). Results with respect to diffusivity parameters were similar when adult-onset and juvenile-onset DM1 patients were compared separately to controls. Zanigni et al. confirmed again the widespread pattern of microstructural damage in DM1 patients, independently if patients with very long CTG repeats were included or not (44).

Only three out of seven studies investigating correlations between CTG repeat length and diffusivity parameters found a correlation. Wozniak et al. reported in their tractography-based study of juvenile/adult-onset DM1 patients an association of CTG repeat length with MD values of the corticospinal tract and cingulum (37). Minnerop et al. described in adult-onset DM1 patients correlations with FA values of association fibers, fornix, cingulum bundles, corpus callosum, and left external capsule (14). Serra et al. described a correlation with FA values across the entire brain, including corpus callosum, brainstem and cerebellum (40). Zanigni et al. found only a correlation, as long as patients with very long repeats were not excluded (44).

Associations between cognitive performance and diffusivity parameters were investigated in nine studies and at least some kind of correlation was reported in seven. In congenital and juvenile-onset DM1 patients, Wozniak et al. found a correlation for whole brain FA values with lower IQ and executive functioning (13) and a ROI-based analysis in childhood/juvenile-onset DM1 patients revealed an association between FA and MD values (in particular of frontal and temporal lobes) with working memory performance (61). Several studies showed in non-congenital DM1 patients correlations between diffusivity parameters and neuropsychological test results. Serra et al. found correlations of generalized FA reductions with MMSE scores (40). Zanigni et al. found correlations between the MMSE score and diffusivity changes within the splenium and the posterior part of the corpus callosum, posterior corona radiate, and posterior thalamic radiations bilaterally and right retrolenticular part of the internal capsule (44).

Cabada et al. found correlations between MD and FA values of the posterior corpus callosum and visuospatial impairment (46). Similar, Baldanzi et al. observed an association between RD and AD values of the corpus callosum and visuomotor coordination and working memory tasks (43). Further associations were detected between visuo-spatial and episodic verbal memory and associative tracts of the internal capsule and coronal radiata. Caso et al. found a correlation between MD values of the left corona radiata and association fibers within the frontotemporal WM regions and orientation and attention scores (38). Working memory performance was correlated with MD values of all tracts investigated by Wozniak et al. while processing speed

was associated with MD values of the corticospinal tract and association fibers (37).

Correlations with the MIRS score were found in three studies (14, 37, 40). Correlations with disease duration or age were investigated by five (disease duration), respectively seven (age), DTI studies, but a correlation between FA values and disease duration or age was only found by one (14). Noticeably, the pattern of affected structures was identical between correlation analysis of age and disease duration. Associations between diffusivity parameters and sleepiness or fatigue were investigated in four studies, but only two found significant correlations (14, 37). FA values in Minnerop et al. were lower in patients with less fatigue and less depressed mood (14). This somewhat contradictory finding of higher FA values associated with more depressed mood was interpreted in line with the work by Winblad et al. (63) as possible hint toward reactive depressed mood in early disease stages and the presence of more effective coping strategies or less abilities to perceive own limitations in later disease stages (associated with lower FA values). Fatigue correlated only minor with FA values of the corpus callosum, but showed instead a correlation with FA values of the brainstem (14). Wozniak et al. found MD values of association fibers related to sleepiness (37).

Functional Brain Imaging in DM1

Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) in DM1

Please see **Table 3** for technical details of the included studies.

The first functional brain imaging study on adult DM1 patients was performed by Fiorelli et al. in 1992 using FDG-PET (15). They found about 20% reduced glucose utilization rate in DM1 patients in comparison to controls. Mielke et al. found reduced uptake in all cortical and subcortical regions, predominantly in frontal regions and lentiform nucleus (64), and Annane et al. confirmed these results and reported a negative correlation between glucose consumption and CTG repeat length and plasma insulin levels, reflecting peripheral insulin resistance (65). Renard et al. reported—albeit not corrected for partial volume correction—bilateral symmetrical reduced FDG uptake in the lateral part of the frontal lobes, affecting most severely Brodmann area 8 (related to eye movement control), while deep GM structures did not show reduced metabolism (67). CTG repeat length did not correlate with hyopmetabolism, although there was a tendency toward lower FDG uptake with CTG repeat length >1000 and early (childhood) onset DM1, congenital cases were not included. Weber et al. also reported widespread and symmetric hypometabolism in the frontal lobes stretching to part of the temporal lobes (33). This finding cannot simply be attributed to cortical atrophy, since results remained unchanged even after partial volume correction in a subgroup. However, no correlations with NPT were found. The most recent FDG-PET by Peric et al. analyzed glucose metabolism and its relation to neuropsychological testing, excluding congenital and late-onset DM1 cases (68). The most prominent hypometabolism was present in prefrontal, frontotemporal, temporal, and precentral regions, subcortical GM regions were less affected than cortical areas. Right frontotemporal hypometabolism correlated with executive dysfunction.

Chang et al. investigated cerebral blood flow and perfusion via SPECT ligands and compared subgroup of DM1 patients with maternal and paternal inheritance (16). Although no congenital cases were included, according to the demographic data at least subjects with childhood onset were included. Blood flow (CBF) and perfusion was reduced compared to controls, particular in the temporal and frontal regions and regional CBF correlated with NPT performances, while the mode of inheritance had significant impact only on perfusion, but not on CBF—most likely due to low resolution of the ^{133}Xe-SPECT measuring CBF. Meola et al. described a marked reduction CBF via H_2O^{15}-PET in orbitofrontal, medial and dorsolateral frontal cortex, temporal pole, hypothalamus and left basal ganglia (22). Reduced temporal and frontal CBF was also reported in two DM1 cases (one with childhood onset) (66). Romeo et al. analyzed retrospectively perfusion (SPECT) in a large group of DM1 patients—but without matched controls (69). A subgroup of patients also received FDG-PET. They described global hypoperfusion (and to a lesser extent reduced glucose metabolism) more pronounced on the left hemisphere, frontal regions, and general in cortical regions more present than in subcortical structures. With respect to cortical regions they also described a hypoperfusion gradient from frontal, parietal, temporal, occipital sensory-motor to insular regions, and frontal hypoperfusion even correlated with MIRS scores.

Proton MR Spectroscopy (^{1}H-MRS) in DM1

Please see **Table 4** for technical details of the included studies.

^{1}H-MRS allows analyzing biochemical properties of the brain *in vivo*. Metabolites commonly measured are N-acetylaspartate (NAA), phosphocreatine (Cr), and choline (Cho). NAA is only present in neurons (neuronal marker), and neuronal loss is usually correlated with decrease of the NAA content. Cho represents a membrane-bound molecule of neuroglial cells and can be regarded as glial marker. Creatine is ubiquitously expressed in brain and reflects the energy potential available in brain tissue. Measuring these metabolites at longer echo time is advantageous regarding signal-to noise ratio: Interference with signals from other metabolites is avoided since their peaks occur at shorter TE and have already decayed. Contrary, applying short TE allows to quantify a spectrum of additional neurochemical alterations of amino acids relevant to neural transmission, cell structure, and cell energy metabolism, e.g., glutamine, glutamate, or myo-inositol, a naturally occurring sugar regarded as an glial-specific marker.

Concentrations of specific metabolites can be measured either by single-voxel spectroscopy, where a single sample volume is analyzed, or multi-voxel spectroscopy where multiple voxels in a single slab of tissue are analyzed (MR spectroscopy imaging). Since estimating absolute NAA levels *in vivo* are difficult to obtain and to avoid bias due to interindividual differences, the content is often measured in relation to other molecules (Cho, Cr), but absolute values are advantageous [for methodological details see (78)].

TABLE 3 | Positron emission tomography (PET) and Single photon emission computed tomography (SPECT) in DM1.

Study	Modality (Tracer)	Estimated parameters	Group size	Age (M ± SD) [years]	Control group	Disease duration (M ± SD) [years]	CTG repeat length (range)	Correlation parameters
Fiorelli et al. (15)	PET (^{18}FDG)	glucose consumption	11	35.3 ± 11.2	+	-	-	Cortical atrophy
Mielke et al. (64)	PET (^{18}FDG)	glucose consumption	3	42/50/59	+	-	-	-
Chang et al. (16)	SPECT (^{133}Xe, ^{99m}TC-HMPAO)	CBF, perfusion	22 (no congenital)	36.6 ± 14.0	+	13.5 ± 6.9	-	NPT (+)
Annane et al. (65)	PET (^{18}FDG)	glucose consumption	11	43 ± 12	+		250–5000	CTG (+), plasma insulin level (+)
Meola et al. (42)	PET (H_2O^{15})	CBF	11	42.7 ± 14.6*	+		500–700*	-
Takeda et al. (66)	SPECT	CBF	2	35/55	-	25/19	1300	-
Romeo et al. (69)	SPECT (^{99m}TC-ECD/HMPAO) PET (^{18}FDG)	perfusion/glucose consumption	58 (+PET: 17)	46 ± 15/ +PET: 49 ± 9	-	12.2 ± 8.6	+	MIRS (+)
Weber et al. (33)	PET (^{18}FDG)	glucose consumption	17 (no congenital/early-onset)	37.2 ± 14.2*	+	16.0 ± 9.6*	-	NPT
Renard et al. (67)	PET (^{18}FDG)	glucose consumption	24 (no congenital)	47 ± 12.5	+	19.1 ± 9.0	83–2000	CTG, age at onset
Peric et al. (68)	PET (^{18}FDG)	glucose consumption	16 (no congenital/late-onset)	45.6 ± 9.6	+	21.8 ± 8.3	-	NPT (+)

*CBF, cerebral blood flow; CTG, CTG repeat length; ^{18}FDG, ^{18}F-Fluordesoxyglucose; MIRS, muscular impairment rating scale; NPT, neuropsychological tests; PET, positron emission tomography; SPECT, single photon emission computed tomography; (+), positive or negative correlation was found; * data refer to whole DM1 study group, including patients that did not undergo PET.*

The first study applying ^{1}H-MRS in five children with congenital DM1 was performed in 1995 by Hashimoto et al. (79). ROIs were placed within right parietal region and in some cases additional ROIs were placed in occipital and frontal regions. Occipital/parietal regions partially included areas with WMHL in two cases. While in matched controls an age-dependent increase of NAA/Cho and NAA/Cr was observed, the NAA/Cho ratio did not correlate with age in DM1 patients and the NAA/Cr on the contrary decreased with age. The Cho/Cr ratio did not differ, showing a decrease with increasing age in both groups. The authors concluded that the decreases in NAA/Cho and NAA/Cr were therefore most likely caused by a decrease of NAA, pointing toward neuronal damage or defective development. A decrease of NAA/Cho and NAA/Cr ratios were found independently of the presence of WMHL in the respective ROI.

Chang et al. reported in a mixed patient group including congenital, juvenile and adult-onset DM1 patients elevated levels of myo-inositol, total creatine, and choline-containing compounds, pointing to an increased glial content in the two brain regions (occipital region, temporoparietal region) studied (70). NAA levels did not differ between DM1 patients and controls. Furthermore, the creatine and myo-inositol peak areas correlated with the CTG repeat length, especially in the temporoparietal brain region.

Akiguchi et al. analyzed in non-congenital DM1 patients NAA/Cho and NAA/Cr in an insular ROI (71). Both ratios were reduced while Cho/Cr did not differ. None of the ratios correlated with age and no difference between patients with and without a subnormal MMSE scores was found. Without measuring absolute values, it remained unclear whether the abnormal ratios were due to reduced NAA (pointing toward neuronal damage) or due to increased Cho or Cr (pointing to increased glial content). But in the light of the study by Chang et al. the authors assumed that their results were reflecting also increased glial content.

Vielhaber et al. estimated in adult-onset DM1 patients absolute values of NAA, Cr and Cho as well as ratios in three ROIs located within midoccipital and temporoparietal GM, but also in frontal WM (72). In contrast to Chang et al. (70), NAA was reduced in all three ROIs and most pronounced in frontal WM, while Cr and Cho were reduced in temporoparietal GM and to a less extent also in the frontal WM. Accordingly, NAA/Cr and NAA/Cho ratios were reduced (13–21 %) while Cho/Cr ratio remained unchanged compared to controls. However, no MRS-parameter correlated with clinical parameters. The authors concluded that glial loss or dysfunction could be a possible cause of the Cr and Cho depletion, while neuronal loss most likely caused the NAA loss. They further postulated that the lack of correlation with age of onset or disease duration argues against primary neurodevelopmental or progressive process in adult-onset DM1.

Takado et al. applied single voxel ^{1}H-MRS within regions of the frontal WM and the anterior cingulate gyrus and MRSI investigating two slices at the basal ganglia or upper lateral

TABLE 4 | Functional brain imaging in DM1.

Study	Modality	Estimated parameters	Group size	Age (M ± SD) [years]	Disease duration (M ± SD) [years]	CTG repeat length (range or M ± SD)	Correlation parameters
Hashimoto et al. (79)	1.5T ^{1}H-MRS (STEAM) TE 270 ms	ROI: parietal (NAA/Cho, NAA/Cr, Cho/Cr)	5 (congenital)	7.3 ± 5.5	congenital	-	Age (+)
Chang et al. (70)	1.5T ^{1}H-MRS (PRESS) TE 30 ms	ROI: midoccipital GM + temporo-parietal GM left NAA, Cr Cho, myoinositol	14	37.8 ± 2.7	13.8 ± 3.5	173–1434	CTG (+), NPT
Akiguchi et al. (71)	1.5T ^{1}H-MRS (STEAM) TE 19 ms	ROI: Insula (NAA/Cho, NAA/Cr, Cho/Cr)	21 (no congenital)	37.0 ± 13.6	11.2 ± 7.6	(+)	Age
Vielhaber et al. (72)	1.5T ^{1}H-MRS (PRESS) TE 135 ms	ROI: frontal WM + midoccipital GM + temporo-parietal NAA, Cho, Cr	14 (no congenital/juvenile onset)	38.8 ± 9.1	-	250–750	Age, age at onset, disease duration, NPT, CTG
Takado et al. (73)	3T ^{1}H-MRS ^{1}H-MRSI (PRESS) TE 30 ms, 144 ms	ROI: ant. cingulate gyrus, frontal WM, Slices BG level, (tNAA, tCho, Cr, MI, Glu, Gln, Glx, NAA/Cho, NAA/Cr, Cho/Cr)	13	43.6 ± 12.6	11.8 ± 9.0	685 ± 462	NPT (+), CTG (+)
Caramia et al. (32)	1.5T fMRI	self-paced sequential finger-to-thumb opposition task (right hand)	15	36.3 ± 12.3	15.4 ± 12	96–1570	Age (+), disease duration, MIRS score, WMHL-load
Toth et al. (74)	3T fMRI	myotonia-inducing grip task prior and after warm-up procedure	16	47.9 ± 8.0	18.3 ± 9.0	+	-
Serra et al. (36)	3T rsfMRI	DMN functional connectivity	27 (no congenital)	39 ± 11.8	-	54–2000	Personality traits/disorders (+)
Serra et al. (80)	3T rsfMRI	theory of mind-network functional connectivity/graph theory	20 (no congenital)	43.9 ± 10.7	-	150–1200	-
Serra et al. (82)	3T rsfMRI	functional connectivity/graph theory	31 (no congenital)	39.9 ± 11.4	-	54–2000	MIRS (+), CTG, NPT (+)
Park et al. (75)	3T rsfMRI	sensorimotor network functional connectivity/power spectral density	18 (adult-onset)	44.4 ± 10.7	13.4 ± 2.0	374 ± 66	Motor performance (+), CTG (+), disease duration (+)
Krogias et al. (76)	ultrasound	echogenicity of basal ganglia/mesencephalic regions, ventricle diameters	17	39 ± 15	17 ± 6	75–1000	Age (+), CTG (+), daytime sleepiness (+), depression
Peric et al. (77)	ultrasound	echogenicity of basal ganglia/mesencephalic regions, ventricle diameters	61 (no congenital)	41.2 ± 10.3	18.9 ± 8.6	747 ± 280	Sex (+), disease duration (+), CTG, MIRS, depression (+) fatigue (+), RLS

All included studies investigated patients in comparison with controls. Cho, choline; Cr, creatine; CTG, CTG repeat length; DMN= default mode network; fMRI, functional MRI; Gln, Glutamin; Glu, Glutamate; Glx, Glu+Gln; GM, gray matter; MI, myo-inositol; MIRS, Muscular Impairment Rating Scale; MRS, magnetic resonance spectroscopy; NAA, N-acetyl aspartate; NPT, neuropsychological tests; PRESS, point resolved spectroscopy (MR pulse sequence); ROI, region of interest; rsfMRI, resting state functional MRI; SE, spin echo technique; STEAM, stimulated echo acquiring method (MR pulse sequence); tCho, glycerophosphcholine + phosphocholine; tNAA, NAA + N-Acetylaspartylglutamate; WMHL, white matter hyperintensive lesions; (+), positive or negative correlation was found.

ventricles level (73). By MRSI they found decreased NAA/Cr ratio in insula cortex, putamen, thalamus, internal capsule (posterior limb), frontal, and posterior WM. Cho/Cr ratio was increased in the thalamus and reduced in FWM. Similar, single-voxel ^{1}H-MRS showed decreased NAA and NAA/Cr in both ROIs (anterior cingulate gyrus and frontal WM), Cho and glutamine (in frontal WM). After correcting for partial volume effects a significant increase in Cho, glutamine, and glutamate concentrations were found in the anterior cingulate gyrus, pointing toward disturbed glutamatergic system with the frontal lobe. NAA/Cr in frontal WM correlated with NPT measures and with the CTG repeat length. The authors concluded that neuronal abnormalities seem to occur, both, in GM and WM, without significant gliosis.

fMRI and Resting-State fMRI in DM1

Please see **Table 4** for technical details of the included studies.

Caramia et al. investigated a self-paced sequential finger-to-thumb opposition task (right hand) and found a greater activation in DM1 patients compared to controls in bilateral sensorimotor areas, inferior parietal lobules, basal ganglia and thalami and ipsilateral premotor area, insula and SMA (32). Further, age correlation was greater in patients than in controls in bilateral sensorimotor areas and in contralateral parietal areas. The authors hypothesized that the increased brain motor activation reflects a compensatory mechanism and because of similarities with alterations found in healthy aging. They postulated that the observed changes are part of an accelerated aging process.

While Caramia et al. (32) tried to avoid any interference of their fMRI-motor task with myotonia, Toth et al. specifically investigated the impact of myotonia on cerebral functioning via fMRI (74). They compared DM1 patients with and without grip myotonia while performing a grip task before and after a warm-up procedure. In patients presenting grip myotonia they found higher activity within the SMA and the dorsal anterior cingulate cortex (ACC). The same regions were also activated when fMRI activity was compared before and after the warm-up session in patients with grip myotonia. No activation of primary motor areas occurred during myotonia, which was interpreted by the authors as further evidence against an involvement of brain function in the development of myotonia. Instead, the observed activity probably reflects compensational cortical activations. SMA is part of active inhibitory circuits which are usually activated preceding voluntary muscle relaxation, so the authors postulated that activation within this region during myotonia may reflect the unconscious intention to finish the abnormally prolonged muscular activation. In turn, ACC links cognitive functions to motor actions and seems to play a role in error detection. So the authors hypothesized that the increased activity within the ACC could be related to the error-detection of experiencing non-occurring grip relaxation despite the intention to do so.

Instead of measuring the BOLD (blood oxygen level—dependent) signal in response to specific task as mentioned above, resting state fMRI is a functional MRI technique investigating fluctuation of the BOLD signal of the brain in rest without performing an active task. Functional connectivity can be estimated between spatially distinct brain regions by identifying temporal synchronous frequency fluctuations between the respective regions. This leads to the identification of specific function- or disease- related networks. One of the most frequently investigated networks is the default mode network (DMN) comprising brain regions which are active in wakeful rest and deactivated when performing active tasks.

Serra et al. investigated for the first time the interaction of personality traits/disorders in DM1 patients and functional connectivity within the DMN (36). Functional connectivity was increased within the bilateral posterior cingulate and left parietal DMN nodes. Additionally, DMN functional connectivity within the left supramarginal gyrus (parietal node) and right putamen and caudate nucleus (inverse correlation) was strongly associated with schizotypal-paranoid traits in DM1 patients. The authors postulated that an overengagement of the DMN may lead to an exaggerated focus on one's own thoughts and feelings. The altered functional connectivity of the basal ganglia might be related to reduced cognitive flexibility in association with schizotypic—paranoid traits, probably accounting for patients rigid thoughts and fixed ideas.

In a consecutive study by the same group (Serra et al.), the authors investigated social cognition with the Theory of Mind (ToM) framework in relation to resting-state functional connectivity (80). The authors applied Graph theory to analyze functional networks. Graph theory-based approaches model the brain as a complex network represented graphically by a collection of nodes, indicating anatomical elements (e.g., brain regions) and edges (e.g., connectivity between nodes). After generating a network model, several metrics can be used to characterize and even quantify network properties on a local and global level (81). Some nodes are more critical (i.e. centrality) for information processing (efficiency in information transferring) and are called "hubs". Serra et al. identified a ToM network containing 14 nodes and 9 edges (80). While global topological properties of the identified ToM network did not differ between DM1 patients and health controls, comparison of local properties showed a significant increase of nodal efficiency and degree in the left inferior temporal gyrus. In DM1 patients, this region was significantly more connected to dorsolateral prefrontal cortex and cerebellum than in controls. In contrast, connections between inferior temporal gyrus and occipital regions were only observed in healthy subjects. The observed deficits within ToM tests in DM1 patients in association with abnormal connectivity between the left inferior temporal and fronto-cerebellar nodes, further underpins that difficulties in social interactions as well as personality traits are related to brain abnormalities and should not be regarded as reactive symptoms.

In another study by Serra et al. resting state fMRI data were analyzed with network-based analysis and graph theory (82). Additionally, correlation analysis between network metrics and clinical data were performed. Dysfunctional hubs were located in the bilateral anterior cingulum, orbitofrontal cortex, and right parahippocampal gyrus. Connectivity correlated with NPT (visuospatial reasoning). With respect to graph theory analysis, no global, but local measures of connectivity differed significantly between groups (nodal degree, betweenness centrality, nodal

efficiency). The anterior pattern with decreased fronto-parietal connectivity could probably be linked to cognitive and behavioral symptoms in DM1 patients. The posterior pattern with increase connectivity in SMA and cerebellum resembles according to the authors to pattern observed in patients with autism spectrum disorders and could represent repetitive stereotyped behaviors observed in autism and autism-like traits have been reported it patients with congenital DM1. Additionally, the regions involved in the posterior pattern are motor-related regions and may be relevant for the motor impairment in DM1 patients, The authors propose that the abnormally high connectivity in these motor regions might represent a compensatory, albeit inefficient or maladaptive mechanism of brain plasticity.

The most recent functional MRI study by Park et al. investigated power spectral density (PSD) in the resting-state sensorimotor network in DM1 patients (75). In contrast to functional connectivity analysis based on the correlation analysis between different brain regions, they applied power spectral density (PSD) analysis. Power spectrum is a physical quantity that can quantitatively reflect energy density (and consumption) changes, e.g., of low-frequency BOLD fluctuations. Compared to controls there was in DM1 patients a decrease of PSD in right superior temporal pole, and bilaterally in middle and inferior temporal gyrus, postcentral gyrus, occipital gyrus, precuneus, posterior cingulate, and cerebellum. An increase of PSD was detected in the orbitofrontal cortex, putamen, parahippocampal gyrus, fusiform gyrus, anterior insula cortex, and pallidum. Higher PDS responses were also found in WM structures (cerebral peduncle, head of caudate nucleus, anterior/posterior limb of internal capsule, externa capsule and cortical association fibers). Correlation analysis revealed an association between brain regions with altered PSD and motor performance, CTG repeat length, and disease duration. The authors concluded that motor disability in DM1 maybe strongly associated with abnormality in the visual processing network and that GM and WM PSD alterations seem to be involved in motor deficits in DM1 patients.

Transcranial Ultrasound in DM1

Please see **Table 4** for technical details of the included studies.

Transcranial B-Mode sonography is well-established in neonatology and has been used in congenital DM1 early to assess brain structure and integrity. Intracranial pathological findings like intracerebral hemorrhage, hydrocephalus or ventricular enlargement can be easily and non-invasively detected by application of this bedside technology. In congenital DM1, cerebral ventricular enlargement has frequently been described in single cases or smaller series of patients (83–85).

Only in recent years, the technique of transcranial sonography has been applied to adult-onset DM1 patients to assess the echogenicity of the brainstem raphe, mesencephalon and substantia nigra as well as the third ventricle width in cross-sectional study designs (76, 77). The authors reported that brainstem raphe hypoechogenicity was more common in DM1 patients than in controls, and both hypoechogenicity and hyperechogenicity of the substantia nigra were more frequent in DM1 patients. Moreover, the width of the third ventricle was increased in DM1 patients compared to controls (77). A second study on adult-onset DM1 and DM2 patients evaluated the echogenicities of basal ganglia and mesencephalic regions as well as ventricle diameters, however did not entirely distinguish between both disease entities. In myotonic dystrophy patients, hyperechogenicity of the substantia nigra and/or hypoechogenicity of the mesencephalic raphe were frequent findings predominantly in DM1. The width of the third ventricle was significantly larger in patients (76).

In conclusion, transcranial ultrasound B-Mode examinations are also feasible in adult-onset DM1 for the measurement of ventricular enlargement and may also detect changes in the echogenicity of specific brainstem structures.

Myotonic Dystrophy Type 2

There are much more neuroimaging studies on DM1 than on DM2. The first brain imaging study on PROMM patients was performed in 1997 (7). Shortly afterwards it was discovered that PROMM and DM2 are naming the same disorder (86). Since then, a thorough Pubmed-database search revealed only further 13 neuroimaging studies and two transcranial sonography studies addressing DM2. The number of patients that were included in MRI studies were always quite low, ranging between one and 22 patients. Findings and conclusions of those studies are partly contradictory. So far, no longitudinal imaging studies on DM2 patients have been published. These are some of the reasons that explain why the natural history of brain alterations in DM2 still remains unclear.

Conventional Morphological Brain MRI in DM2

Please see **Table 5** for technical details of the included studies.

Earlier studies used 1.0 or 1.5 T MRI for routine brain imaging on DM2 patients, and their main common findings were diffuse periventricular WMHL (**Figure 1C**) (7, 22, 24, 31, 33). From these earlier studies and those performed later using 3T-MRI (14, 41) it can be concluded that the amount of WMHL in DM2 patients is less compared to DM1 and that they are located predominantly in frontal but also in parieto-occcipital brain regions (**Figure 1C**). Temporal WMHL seem to be restricted to DM1 (14, 24, 31). There are contradictory assumptions regarding the lesion load, whether the lesions are confluent or non-confluent. Whereas Meola et al. and Romeo et al. did not reveal associations between WMHL and cognitive testing or neuromuscular impairment or age (22, 31), Weber et al. described that WMHL correlated with psychomotor speed (33).

Structural Brain Imaging in DM2

Quantification of Global Brain Volume in DM2

Please see **Table 6** for technical details of the included studies.

Using routine 1.5 T brain MRI, Kornblum et al. described brain atrophy as prominent feature in DM2. Other studies also addressed this issue by applying BPF (33, 52, 87) and CNS volumetry (41). Kassubek et al. found only slightly decreased BPF in DM2 patients (52), while others (14, 33, 41), partly including larger patient groups and improved hardware, reported significant brain atrophy in DM2 patients compared to controls. However, their conclusions

TABLE 5 | Conventional MRI in DM2.

Study	Modality	Estimated parameters	Group size (MRI done)	Age (M ± SD or range) [years]	Age at onset [years]	Control group	Disease duration (M ± SD or range) [years]	Correlation parameters
Hund et al. (7)	1.0T, 1.5T (T1, T2)	WMHL	10 (9)	27–64	26–58	-	-	-
Meola et al. (42)	1.5T (T1, PD, T2)	GCA, FCA, WMHL	20 (17)	18–73	decade 1–4	+	-	Age, NPT, neuro-muscular involvement
Kornblum et al. (24)	1.5T (T1, T2, FLAIR)	atrophy, WMHL	9 (9)	42–68	20–55	-	2–19	-
Romeo et al. (31)	1.0T T1,T2, FLAIR,DWI	WMHL	14 (12)	28–71*	5–67	+	16.3 ± 10.2*	NPT, neuro-muscular involvement
Weber et al. (33)	1.5T (T1, T2, FLAIR)	WMHL	9 (9)	53.4 ± 10.9	-	+	23.0 ± 15.0	Age, disease duration, NPT (+)
Minnerop et al. (11)	3T (T2)	WMHL	22 (22)	52.5 ± 10.1	-	+	11.9 ± 9.9	-
Schneider-Gold et al. (41)	3T (FLAIR, T1)	WMHL, VRS	16 (15)	52 ± 7*	24–49	+	14 ± 9*	Other clinical CNS symptoms

*T1, T1-weighted MR sequence; T2, T2-weighted MR sequence; PD, Proton-density-weighted MR sequences; WMHL, white matter hyperintensive lesions; FCA, Focal cerebral atrophy; GCA, Global cerebral atrophy; VRS, Virchow-Robin-spaces; (+), positive or negative correlation was found; * data refer to whole DM2 study group, including patients that did not undergo MRI.*

TABLE 6 | Quantification of brain volume in DM2.

Study	Modality	Estimated parameters	Group size (MRI done)	Age (M ± SD) [years]	Age at onset [years]	Disease duration (M ± SD) [years]	Correlation parameters
Kassubek et al. (52)	1.5T (T1)	BPF	9 (9)	53 ± 11	-	26 ± 16	Age (+), disease duration, motor score, educational level
Minnerop et al. (87)	1.5T (T1)	BPF VBM (WM, GM), SBM (callosal thickness)	13 (13)	53.3 ± 12.0	-	12.0 ± 8.8	-
Weber et al. (33)	1.5T (T1, T2, FLAIR)	BPF, VBM (GM)	9 (9)	53.4 ± 10.9	-	23.0 ± 15.0	Age (+), disease duration, NPT (+)
Minnerop et al. (11)	3T (T1)	VBM (GM, WM)	22	52.5 ± 10.1	-	11.9 ± 9.9	-
Franc et al. (57)	3T (T1)	DTI (GM volume)	5 (5)	38–49	29.5	-	Masticatory muscle, FA decrease (+)
Schneider-Gold et al. (41)	3T (T1)	VBM (GM, WM), volumentry (total GM/WM, cerebellum, brainstem, upper cervical cord, ventricle)	16 (15)	52 ± 7*	24–49	14 ± 9*	NPT (+), depression (+), daytime sleepiness (+), MIRS

*All included studies investigated patients in comparison with controls. BPF, brain parenchymal fraction; CSF, cerebrospinal fluid; CT, cortical thickness; DTI, diffusion tensor imaging; GM, gray matter; MIRS, muscular impairment rating scale; NPT, neuropsychological tests; SBM, surface-based morphometry; VBM, voxel-based morphometry; WM, white matter; (+), positive or negative correlation was found; * data refer to whole DM2 study group, including patients that did not undergo MRI.*

whether brain atrophy was driven by GM atrophy (33, 41) or WM decrease (14) remained contradictory. Kassubek et al. did not find any significant correlations of BPF in DM2 to clinical parameters (disease duration, motor score, and educational level) (52). In contrast, Weber et al. found a strong correlation of BPF to age and to visuo-constructive abilities and psychomotor speed, albeit less significant (33).

Quantification of Regional Brain Volume in DM2

Please see **Table 6** for technical details of the included studies.

For quantitative neuroimaging analysis a number of studies applied VBM. Minnerop et al. and Weber et al. performed VBM analyses using 1.5 T MRI in 13 and 9 DM2 patients, respectively, compared to controls (14, 87). They found cortical GM reduction, but also subcortical GM reduction in hypothalamus, thalamus, brainstem, and adjacent midline

brain regions. Surprisingly, in a later study by Minnerop et al. performing VBM analyses using 3.0 T MRI in a larger group of DM2 patients no GM decrease was detected (14). Since mean age of DM2 patients was very similar across these studies, this discrepancy might be attributed to different sample sizes. However, VBM analyses revealed predominant WM alterations along corpus callosum and in every lobe, but also in the cerebellum (14, 87).

In contrast to these findings, Schneider-Gold et al. applying VBM in 16 DM2 patients in comparison to DM1 patients and controls observed the opposite relation of GM and WM effects, with a more pronounced GM loss, affecting cuneus, temporal regions, and amygdala (41). They revealed WM atrophy in the cingulate and in the subgyral WM of the medial frontal cortex and primary somatosensory cortex.

Weber et al. found hippocampal atrophy that correlated to deficits of nonverbal episodic memory in DM1 and DM2 (33). Contrary to these findings, Schneider-Gold et al. performed a range of neuropsychological tests but observed significant correlations only between flexibility of thinking and GM volume of the periaqueductal GM, midbrain, thalamus, parahippocampal gyrus, and anterior cingulate (41). Moreover, excessive daytime sleepiness was associated with GM reduction in the mediofrontal cortex and with WM reduction in the middle cerebellar peduncles and parts of pons/midbrain. Depression was associated with brainstem atrophy.

Quantification of White Matter Alterations in DM2

Please see **Table 7** for technical details of the included studies.

DTI was applied in some DM studies in order to analyze the microstructural WM integrity. Minnerop et al. revealed WM decrease throughout the brain in DM2 patients (14). Corpus callosum was mainly affected, but also other association and projection fibers, such as internal and external capsules, and also the limbic system (fornix, cingulate bundle). However, WM decrease was less in DM2 compared to DM1 patients. Correlation analyses showed associations with age, disease duration and motor performance, also depressed mood and fatigue were associated with WM alterations in DM2. In contrast to this, no significant correlations were found between WM integrity and neuropsychological performance in DM2. In a DTI-study by Franc et al. (57) the brain was divided into compartments (supra-callosal, superior-frontal, inferior-frontal, occipital compartments). But pair-wise analyses between DM2 patients ($n = 5$) and controls did not reveal significant differences, in contrast to DM1 patients.

Functional Brain Imaging in DM2

Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT) in DM2

Please see **Table 8** for technical details of the included studies.

The first functional brain imaging study on DM2 patients was performed by Meola et al. in 1999 using H_2O^{15}-PET to analyze the regional cerebral blood flow (rCBF) (22). They revealed a reduced rCBF in DM2 patients in the orbitofrontal and medial frontal cortex and discussed that this might be in accordance with deviance of behavior in DM patients.

Analyses of cerebral glucose metabolism by Weber et al. using FDG-PET on 9 DM2 patients showed significant widespread hypometabolism in frontal lobes stretching to part of the temporal lobes (33). In this study, the pattern of GM decrease (VBM) was compared to the pattern of hypometabolism (FDG-PET). Interestingly, as patterns were not conforming, it was concluded that the hypometabolism was an independent phenomenon of the disease and not a result of GM atrophy. Moreover, they did not find significant correlation of hypometabolism to neuropsychological results.

In contrast to this, a recent FDG-PET study by Peric et al. on 13 DM2 (and 16 DM1) patients observed numerous correlations with neuropsychological test results (68). They detected hypometabolism in pericentral, prefrontal, temporal regions, but also in insula, thalamus, and striatum. In DM2 they observed associations between attention deficit and prefrontal, insular and striatal hypometabolism. Executive dysfunction was associated with prefrontal and insular, right parietotemporal and frontotemporal hypometabolism.

A case report by Sansone et al. performing PET in a DM2 patient with parkinsonism revealed hypometabolism in the posterior thalamus and it was concluded, that parkinsonian features in DM2 are not a result of neurodegeneration of the nigrostriatal system but rather of hypometabolism in the posterior thalamus (89).

A neuroimaging study using single photon emission computed tomography (SPECT) by Meola et al. revealed in accordance to the PET studies, hypometabolism in frontal brain regions, but also in parietooccipital cortical regions (88).

In contrast to earlier mentioned functional brain imaging studies, Romeo et al found most significant hypoperfusion in (left) parietal lobes using perfusion SPECT on 9 DM2 patients (69).

Proton MR Spectroscopy (^{1}H-MRS) in DM2

Please see **Table 8** for technical details of the study.

Vielhaber et al. used ^{1}H-MRS on 15 DM2 patients, analyzing occipital and temporoparietal cortical regions and subcortical frontal WM (72). Compared to healthy controls, they revealed reduced metabolism in all tested brain regions.

Transcranial Ultrasound in DM2

Please see **Table 8** for technical details of the included studies.

In 2015 two studies using transcranial sonography on DM2 patients were published. The drawback of the study by Krogias et al. (76) was, however, that results of DM1 ($n = 17$) and DM2 ($n = 14$) patients were put together, not distinguishing entirely between both disease types. Compared to controls, transcranial sonography analyses in these patients revealed hypoechogenic signal in mesencephalon raphe and hyperechogenic signal in the substantia nigra in 29% of the patients. Looking at the separate results of both groups reveals that these sonography findings are much more frequent in DM1 than in DM2 (results for DM2: substantia nigra hyperechogenic 14.3%; mesencephalic raphe hypoechogenic

TABLE 7 | Quantification of white matter alterations in DM2.

Study	Modality	Estimated parameters	Group size	Age (M ± SD) [years]	Age at onset [years]	Control group	Disease duration (M ± SD) [years]	Correlation parameters
Minnerop et al. (11)	3T DTI	DTI-TBSS (FA, MD, RD, AD)	22 (22)	52.5 ± 10.1	-	+	11.9 ± 9.9	Age (+), disease duration (+), motor performance (+), depression (+), fatigue (+), NPT
Franc et al. (57)	3T DTI	DTI (FA), ROI-based	5 (5)	38–49	29.5	+	-	Masticatory muscle volume (+)

AD, axial diffusivity; DTI, diffusion tensor imaging; FA, fractional anisotropy; MD= mean diffusivity; NPT, neuropsychological tests; RD, radial diffusivity; ROI, region of interest; TBSS, tract-based spatial statistics; (+), positive or negative correlation was found.

TABLE 8 | Functional brain imaging in DM2.

Study	Modality	Estimated parameters	Group size (imaging done)	Age (M ± SD) [years]	Age at onset [years]	Control group	Disease duration (M ± SD) [years]	Correlation parameters
Meola et al. (42)	H_2O^{15}-PET (resting state), SPECT	rCBF	20 (10)	18–73	decade 1–4	+	-	-
Meola et al. (88)	SPECT	CBF	19 (5)	49 ± 18	decade 2–3	+	-	-
Sansone et al. (89)	-PET (^{18}F-FDG, ^{11}C-β-CIT-FE, ^{11}C-raclopride)	glucose metabolism, presynaptic dopamine reuptake, postsynaptic D2 receptor density	1 (1)	72	68	-	5	-
Vielhaber et al. (72)	1,5T Proton MRS	cerebral metabolism (NAA, Cho, Cr)	15 (15)	38.6 ± 7.8	>18	+	9.5 ± 6.8	-
Romeo et al. (69)	SPECT	CBF	14 (9)	54 ± ?	5–67	-	-	-
Weber et al. (33)	FDG-PET	glucose metabolism	9	53.4 ± 10.9	-	-	23.0 ± 15.0	-
Krogias et al. (76)	TCS	basal ganglia, mesencephalic regions	14 (14)	50 ± 7	9–49	+	13 ± 8	-
Rakocevic-Stojanovic et al. (90)	TCS	substantia nigra, brainstem raphe	40 (40)	51.4 ± 10.6	37.4 ± 11.1	+	14.6 ± 13.3	-
Peric et al. (68)	18F-FDG-PET	glucose metabolism	13 (13)	51.8 ± 8.4	36.5 ± 7.1	-	15.3 ± 10.5	NPT (+)

Cho, choline; Cr, creatine; ^{11}C-β-CIT-FE, ^{11}C-N-(2-fluoroethyl)-2 beta-carbomethoxy-3 beta-(4-iodophenyl)nortropane; ^{18}FDG, ^{18}F-Fluordesoxyglucose; fMRI, functional MRI; MRS, Magnet resonance spectroscopy; PET, positron emission tomography; (r)CBF, (regional) cerebral blood flow; NAA, N-acetyl aspartate; SPECT, single photon emission computed tomography; TCS, transcranial sonography; (+), positive or negative correlation was found.

7.1%). There was a correlation between the pathological raphe signal and excessive daytime sleepiness. Moreover, they found a significant enlargement of the third ventricle in DM2 patients compared with controls.

Rakocevic-Stojanovic et al. performed transcranial sonography studies on 40 DM2 patients (90). They revealed higher frequencies of brainstem raphe hypoechogenicity and substantia nigra hyperechogenicity and increased diameter of the third ventricle (DTV). Statistical analyses revealed no correlation of substantia nigra pathology with tremor or bradykinesia in DM2 patients, and no associations of substantia nigra with depression or fatigue. In contrast to this, brainstem raphe hypoechogenicity was associated with fatigue and excessive daytime sleepiness (EDS). DTV was associated with depression and EDS. But, aberrations of brainstem raphe, substantia nigra and DTV did not correlate with sociodemographic or clinical features of DM2.

CONCLUSION

DM1

Please see **Table 9** for a summary of the described regional imaging findings in DM1. Conventional MRI in DM1 patients reveals characteristic findings (e.g., atrophy, ATWML, thin corpus callosum) in particular of WM. However, only by applying more advanced and observer-independent MR methods the true extend of alteration of both WM and GM were perceived. Since then, the dependence of age at onset, the spatiotemporal evolution of brain affection, - are WM and GM changes development-related or neurodegenerative or a mixture of both—and their relevance for clinical symptoms have still been a matter of debate. Up to now, only cross-sectional studies have been published. Thus, many of the relevant questions cannot be sufficiently answered with the study results yet obtained.

VBM studies show a consistent widespread cortical involvement of GM affecting all lobes and in particular

TABLE 9 | Summary of main regional brain changes in DM1 and DM2.

Imaging Modality	DM1	DM2
T1-weighted MRI	• Skull: cranial hyperostosis • Micorcephaly (congenital DM1) • Global atrophy (cortical, hippocampus, basal ganglia) • Ventricular enlargement	• Global atrophy • Ventricular enlargement
T2-weighted MRI	• WMHL (frontal and temporal lobes) • Anterior temporal white matter hyperintense lesions (ATWML) • Dilated Virchow-Robin spaces • Thinning of callosal body (congenital DM1)	• Symmetrical WMHL (periventricular, frontal, parietooccipital) • No ATWML • No dilated Virchow-Robin spaces
VBM/cortical thickness–gray matter reduction	• Cortical (all lobes) (incl. pre- and post-central gyrus, cingulate cortex, hippocampus) • Subcortical (striatum, thalamus, nucleus accumbens, cerebellum, ventral diencephalon)	• Cortical (incl. frontal, temporal, lingual gyrus, cuneus) • Subcortical (brainstem, thalamus, hypothalamus, mesencephalon, int. pallidum, amygdala)
VBM–white matter reduction	• Subcortical in all lobes • Corpus callosum • Fornix • Cingulum bundle • Subcortical (pontine, middle cerebellar peduncle, cerebellum)	• Corpus callosum • Subcortical in all lobes • Cerebellum • Cingulate
T2-relaxometry/ MTI	↑ relaxation times /↓ MT ratios: • WMHL>NAWM • Subcortical (striatum, thalamus)	-
DTI	• WMHL>NAWM • Corpus callosum • Association fibers • Limbic system tracts (fornix, cingulum bundle) • Projection fibers (internal/external capsules, corticospinal tracts) • Brainstem • Cerebellum	• WMHL>NAWM • Corpus callosum • Association fibers • Internal and external capsules • Limbic system (fornix, cingulate bundle)
PET/SPECT	↓ *FDG-Uptake*: • Cortical (frontal>temporal) • Lentiform nucleus ↓ *CBF*: • Cortical (frontal>temporal) • Hypothalamus • Basal ganglia	↓ *FDG-Uptake:* • Frontal > temporal lobes • Pericentral regions • Parietal operculum • Thalamus, striatum ↓ *CBF*: • Frontal, orbitofrontal, parietooccipital cortex
MR-Spectroscopy	↓ *NAA (neuronal loss)* • In WMHL und NAWM • Cortical (frontal, temporo-parietal, occipital, cingulate) ↑*Cr, myo-inositol, Cho (↑glial content)* • Cortical (occcipital, temporoparietal) ↓*Cr, Cho (glial loss)* • Cortical (temporoparietal, frontal)	↓ *NAA* • Cortical (occipital, temporoparietal frontal) *Cr, Cholin* • No difference compared to controls
Ultrasound	• Hypoechogenicity: brainstem raphe • Hyperechogenicity: substantia nigra • Increased width of third ventricle	• Hypoechogenicity: brainstem raphe • Hyperechogenicity: substantia nigra • Increased width of third ventricle

CBF, cerebral blood flow; Cho, choline; Cr, creatine; DTI, diffusion tensor imaging; ^{18}FDG, ^{18}F-Fluordesoxyglucose; MT, magnetization transfer; MTI, magnetization transfer imaging; NAA, N-acetyl aspartate; NAWM, Normal appearing white matter; PET, positron emission tomography; SPECT, single photon emission computed tomography; VBM, voxel-based morphometry; WMHL, white matter hyperintensity lesions.

sensorimotor areas as well as hippocampus. Volume reductions of subcortical GM were present in striatum, thalamus and cerebellum. VBM and DTI studies investigating WM alterations found also a widespread involvement of association fibers, anterior and posterior limb of internal capsule (including the corticospinal tract), external capsule, corpus callosum, cerebellum, and brainstem.

It is well known that brain abnormalities in congenital-onset DM1 differ significantly from changes observed in patients with an adult-onset: Corpus callosum atrophy was already well known for children with a congenital disease-onset, while only by advanced MR techniques the constant and entire involvement of this structure in adult patients was recognized. Unfortunately, most of the previous studies mixed patients with different types of disease-onset. This impedes the delineation of age at onset-related patterns. Furthermore, early-onset leads to an inevitable interference with normal brain development, so it is hardly possible to distinguish pure disease-related brain changes from more secondary changes due to impaired normal brain development. Another issue is the subjective character of the

clinical parameter "disease-onset" since it highly depends on the awareness of patients and their relatives to recognize disease-related symptoms at all.

In spite of these limitations a few studies compared subgroup of patients (38, 44, 57). They observed more pronounced volume reduction of GM in adult-onset DM1 patients than in patients with congenital disease-onset (38, 57), while WM alterations did not differ. Zanigni et al. even observed dissociation between cortical and subcortical GM (44): while the extent of WM alterations and subcortical GM changes remained unchanged after exclusion of patients with a congenital/childhood-onset, cortical GM changes were less pronounced than for the entire group. It could be therefore hypothesized, that the WM involvement occurs early and might be developmental, while with respect to GM involvement aging seems to play a role, pointing toward a neurodegenerative component.

Correlation analysis with CTG repeat length may be another option to obtain insights into this issue, since it is known as a relevant factor regarding disease-onset. Very long repeats (>1000) are usually associated with congenital DM1. However, measuring repeat lengths in blood as biomarker for disease severity in DM1 is highly controversial since they may not correlate with repeat lengths in brain. Somatic mosaicism of repeat expansion may lead to considerable tissue variation in repeat sizes and repeat sizes may even increase throughout life. Nevertheless, it is well accepted that the age at onset and disease severity in DM1 significantly correlate with the number of CTG repeats at least in patients with very high and very low repeat expansions.

However, although frequently performed, correlations with CTG repeat length are often negative and only observed in few studies investigating GM or WM alterations (14, 37, 40, 56). If any correlations with CTG repeat length were detected at all, it was associated with motor-related and frontal areas, further underlining the assumption that central motor areas may contribute to the motor impairment seen in DM1 patients.

Next to structural abnormalities, analyzing functional aspects of the brain is highly relevant for assessing the clinical impact and relevance to observed symptoms. PET and SPECT studies confirm the widespread cortical alteration as well as MRS studies, pointing toward neuronal and glial alterations. Correlation analyses with clinical parameters in these studies however gave heterogeneous results. The fMRI study investigating self-paced sequential finger-to thumb opposition task (32) interestingly lead to an activation of those motor-related regions that had in other DM1-studies been shown to undergo structural changes. Compensatory activations in these regions were only seen in another fMRI-study (74), analyzing the central correlates during myotonia.

The patterns in correlation analyses with other clinical parameters in structural and functional imaging studies are diverse and with the exception of few findings less reproducible. This is in particular true for correlation analysis with neuropsychological test results. Visuo-spatial functions seem to be linked to alterations of the corpus callosum and occipital areas (43, 46). Sleepiness/fatigue was linked with the volume of the ventral diencephalon (46), FA values in the brainstem (14), or MD values within association fibers (37). The heterogeneity may support the current point of view, that the clinical impairments cannot be simply linked to specific and regionally circumscribed structural or functional alterations within the brain. It seems more convincing that disturbed networks build the functional and structural substrate of clinical symptoms observed in DM1 as already seen in other neuropsychiatric diseases. Consecutively, structural and functional network analyses revealed altered functional connectivity within the default mode network in relation to personality traits/disorders in DM1, altered Theory of Mind network in relation to social cognition and detection of connectivity patterns observed in autism spectrum disorders and impaired sensorimotor networks associated with motor performance, CTG repeat length and disease duration (36, 75, 80, 82).

DM2

Please see **Table 9** for a summary of the described regional imaging findings in DM2. Routine brain MRI studies on DM2 showed primarily periventricular WMHL, and no temporal WMHL, in contrast to DM1. Further analyses revealed general brain atrophy. Most VBM studies on DM2 detected GM decrease in various cortical and brainstem regions. DTI imaging verified affected microstructural integrity predominantly of the corpus callosum, but also of numerous other association and projection fibers, including the limbic system. Overall, most of the imaging studies suggest a predominant WM disease, however data are partly contradictory. Functional brain imaging studies showed reduced perfusion and hypometabolism mainly in frontal regions, but also in temporal and parietal/parietooccipital regions. This might be associated to deficits in visuospatial and memory function and avoidant personality trait (88). Some imaging studies compared their results to clinical and neuropsychological data. The assumptions regarding clinical data (age, disease duration etc.) are contradictory. There seems to be a correlation of depression and daytime sleepiness. However, most structural MRI studies deny a correlation of brain affection and neuropsychological performance or find only few correlations with neuropsychological performance. The study by Peric et al. suggests that functional MRI might be more suitable to detect correlations of brain alterations and neuropsychological results (68). TCS studies support earlier findings of brain alterations in the brainstem and enlargement of the third ventricle.

Overall, results of these cross-sectional studies are not very consistent. Future studies need to look at a larger sample of patients. Dealing with a rare disease with diverse prevalences across different countries, multicenter studies would be needed. However, it is a big challenge to examine patients at different centers using the identical hard- and software (e.g., MRI). Moreover, no longitudinal neuroimaging studies on DM2 patients have been published so far. Thus, the natural history of brain involvement in DM2 is still unclear.

Taken together, future imaging studies in DM1 and DM2

- Should establish standard imaging procedures to facilitate the comparability across different study sites [e.g., implementation

of lesion refilling tools in the VBM pipeline to avoid misclassifications of tissue classes during the segmentation step, (91)]
- Should investigate brain alterations with a multimodal approach to produce a comprehensive and versatile view of this complex and variable disorder
- Should include for DM1—if feasible—different ages at disease onset including congenital DM1 to analyze disease-related changes as a continuum
- Should have a longitudinal design to understand whether the across all studies stable and robust finding of widespread WM and GM alterations are the consequence of developmental disturbances, neurodegeneration or both
- Should clearly link functional or structural brain alterations to clinical impairment, thus facilitating the development of biomarkers for upcoming therapeutic studies

AUTHOR CONTRIBUTIONS

All authors contributed to data collection, summary and discussion of intellectual content, manuscript writing, and editing. All authors read and approved the submitted version.

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Distribution and Structure of DM2 Repeat Tract Alleles in the German Population

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Autosomal dominant inherited Myotonic dystrophy type 1 and 2 (DM1 and DM2) are the most frequent muscle dystrophies in the European population and are caused by repeat expansion mutations. For Germany cumulative empiric evidence suggests an estimated prevalence of DM2 of roughly 9 in 100,000, therefore being as prevalent as DM1. In DM2, a $(CCTG)_n$ repeat tract located in the first intron of the *CNBP* gene is expanded. The CCTG repeat tract is part of a complex repeat structure comprising not only CCTG tetraplets but also repeated TG dinucleotides and TCTG tetraplet elements as well as NCTG interruptions. Here, we provide the distribution of normal sized alleles in the German population, which was found to be highly similar to the Slovak population. Sequencing of 34 unexpanded healthy range alleles in DM2 positive patients (heterozygous for a full expansion) revealed that the CCTG repeat tract is usually interrupted by at least three tetraplets which according to current opinion is supposed to render it stable against expansion. Interestingly, only the largest analyzed normal allele had 23 uninterrupted CCTGs and consequently could represent an instable early premutation allele. In our diagnostic history of DM2 cases, a total of 18 premutations were detected in 16 independent cases. Here, we describe two premutation families, one with an expansion from a premutation allele and the other with a contraction of a full expansion down to a premutation allele. Our diagnostic results support the general assumption that the premutation range of unstable CCTG stretches lies obviously between 25 and 75 CCTGs. However, the clinical significance of premutation alleles is still unclear. In the light of the two described families we suggest incomplete penetrance. Thus, as it was proposed for other repeat expansion diseases (e.g., Huntington's disease), a fluid transition of penetrance is more likely rather than a clear cut CCTG number threshold.

Keywords: DM2, intergenerational contraction, *de novo* expansion, premutation, penetrance, prevalence

INTRODUCTION

Myotonic dystrophy type 2 (DM2; PROMM; Ricker syndrome, MIM 602668) is an autosomal dominant multisystemic neuromuscular disorder that occurs in adults (1, 2). Although DM2 and DM1 share the same core features (progressive muscle weakness and atrophy, myotonia, cataracts) as well as additional symptoms like muscle pain, cardiac arrhythmias, endocrinologic disturbances and hypersomnia, apparent differences exist. The disease course is usually more variable and milder

in DM2 with paucisymptomatic cases (3). Brain involvement is rare, there is no congenital form as well as evident anticipation, but myalgic pain is a frequent symptom and muscle weakness is mainly proximal. The frequency of DM2 is highest in Middle Europe.

DM2 is caused by the expansion of an unstable $(CCTG)_n$ repeat located in the first intron of the *CNBP* (cellular retroviral nucleic acid-binding protein) gene in the 3q21.3 chromosomal region (4). Like in DM1 the underlying pathophysiology is a toxic RNA gain-of-function mechanism. The pre-mRNA contains the repeat expansion and remains unprocessed (5). It disrupts cellular pathways like RNA splicing, localization and translation through sequestering of important RNA binding proteins. In muscle fibers insoluable ribonuclear foci are formed. As a result, developmentally inappropriate protein isoforms are expressed in adult tissue.

The complex *CNBP* repeat tract is generally described as $(TG)_n(TCTG)_n(CCTG)_n$. Normal alleles as a rule have <25 copies of the decisive CCTG repeat, whereas expanded alleles have as many as 75–11,000 copies (4). However, the clinical significance of the presumed premutation repeat range between 25 and 75 CCTGs is still unclear and subject of discussion.

In normal sized alleles the CCTG repeat tract is usually found to be interrupted by one or more tetraplets $[(NCTG)_n]$, resulting in more or less stability (4, 6, 7). Consequently, the combined repeat tract in those healthy range alleles can be described by five distinct repetitive motifs: $(TG)_v(TCTG)_w(CCTG)_x(NCTG)_y(CCTG)_z$. In contrast, unstable expanded alleles obviously have a pure CCTG stretch without the NCTG interruption making them more susceptible to strand slippage and unequal crossing over processes in cell division (6) and favoring unusual secondary structures (8).

The composition of the complex repeat motif and allele frequency of normal and expanded alleles has already been studied in the Slovak and American population (6, 7). So far, no equivalent analysis of the *CNBP* repeat motif has been conducted for the German population. In the present report, an estimate of the DM2 prevalence in Germany is given and a statistical analysis of the normal allele length frequency and distribution was performed. Alleles of the most frequent repeat lengths ($n = 31$) as well as of the longest normal alleles ($n = 3$) were sequenced to determine the detailed motif composition and its variance within the German population. Furthermore, we present two small families, one with a transgenerational contraction into the premutational range and another with a transgenerational expansion of a premutation allele to a full expansion.

MATERIALS AND METHODS

Fragment Analysis Data

Fragment analysis by capillary electrophoresis was used to identify the length of unexpanded normal alleles. Data on the repeat length from 739 DM2 negative German probands were collected as a result of routine diagnostic fragment analysis, and statistically analyzed for allele frequencies. Patients' data were anonymized. Genomic DNA was isolated from leukocytes and extracted using standard laboratory procedures. Fragment analysis was performed as previously described (4) with minor modifications. Briefly, the method comprises PCR amplification of the *CNBP* repeat sequence, using a fluorescently marked 6-F-primer (DM2-Cl3N58-F) 5′-GGC CTT ATA ACC ATG CAA ATG-3′, binding 47 bp upstream of the TG repeat sequence. The reverse primer (DM2-Cl3N58-R) 5′ GCC TAG GGG ACA AAG TGA GA 3′ binds directly after the last single TCTG tetraplet following the repetitive sequence (**Figure 1**). Up to 40 CCTG repeats can be amplified with this method. The PCR cycle program consists of the following five steps: Step 1: 95°C (5 min), step 2: 95°C (30 s), step 3: annealing at 56°C (30 s), step 4: elongation at 72°C (30 s), and step 5: final elongation at 72°C (3 min). Steps 2–4 were repeated in 30 rounds. After DNA amplification, 15 µl Hi-Di™ formamide (Thermo Fischer) and 0.5 µl of the MapMarker® 1000 X-Rhodamine (BioVentures) was added to 1 µl of the PCR product and followed by capillary electrophoresis on an Applied Biosystems 3130/3130xl Genetic Analyzer. Genemapper 4.0 was used to analyze the data. For statistical analysis, the combined repeat tract lengths of each allele observed in fragment analysis were calculated by subtracting 92 bp of specific sequence surrounding the *CNBP* repeat from the measured amplicon length. For illustration the *CNBP* repeat sequence including the combined repeat tract length and primer binding sites are depicted in **Figure 1**. The statistical analysis of the frequencies and distribution of the different allele lengths in the German population was done with MS Excel (Office Suite 2016). Please note, that the normal alleles of DM2 positive were not included in the statistical analysis of healthy range repeat sizes. Confirmation of DM2 positive patients was done by RP-PCR (repeat primed PCR) and Southern blotting in routine diagnostics [protocol in (9, 10)].

Sanger Sequencing

Sanger sequencing was conducted in order to characterize *CNBP* repeat motif compositions of the three most frequent allele lengths as well as the longest normal alleles in the German population. Since the length and composition of the repeat motif varies on each allele, Sanger sequencing of blood DNA is challenging in most cases due to overlapping and shifted sequences from two different alleles which impedes a proper sequence read out. Therefore, instead of sequencing probands with homozygous or heterozygous allele lengths in the normal range, 34 DM2-positive patients with full expansion on one allele were sequenced. By reducing PCR elongation time to a minimum of only 2 s, amplification was strongly biased toward the short normal repeat allele rather than the expanded allele. The application of a discriminating PCR protocol allowed the detailed determination of a single allele's sequence avoiding co-amplification of the expanded allele and thereby any overlapping background from a second allele. The PCR primers for sequencing of healthy range length alleles have previously been described by Radvanszky et al. (7). Their binding sites within the *CNBP* sequence are depicted in **Figure 1** in italics. In detail, the applied touchdown PCR protocol comprised the following annealing temperature steps: 62°C (30 s, 2 cycles), 59°C (30 s, 2 cycles), and 56°C (30 s, 30 cycles), each followed by an elongation step for only 2 s at 72°C, and a final elongation step of 5 min. PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara, CA) treatment.

*TCCTCACTCCGAGAATCC*ATTTTACAGCTTCATTGGTTTGGGTTATTCCAATTTTT
TGATGTGAGTAAATAAATGACTTCTATTTGCCCAAAATAAAGCTTATATAGGCCTT
ATAACCATGCAAATGTGTCCATTAAGTTGGACTTGGAATGAGTGAATGAGTATTAC
TGCCAG **(TG)$_v$(TCTG)$_w$(CCTG)$_x$(NCTG)$_y$(CCTG)$_z$**TCTGTCTCACTTTGTCCCC
TAGGCTGGAGTGCAGTGGTATGATCTCGGCTCACTGCAACCTCCACCCCCCGG*GTT*
CAAGCGATTCTTCTGCC

FIGURE 1 | The combined *CNBP* repeat tract and its surrounding specific sequence with indicated primer binding sites for PCR amplification. Primers for fragment analysis are highlighted in gray and indicate the fragment analysis amplicon. Primer binding sites for sequence analysis from Radvanszky et al. (7) are indicated in italics. The combined repeat tract is denoted in bold. For statistical analysis, the specific combined repeat tract lengths were calculated by subtracting 92 bp of specific sequence surrounding the *CNBP* repeat (68 bp upstream and 24 bp downstream the bold repeat tract) from the measured amplicon length by fragment analysis.

In order to get an impression of the heterozygosity of the locus, 11 DM2 negative patients that appeared to be homozygous due to a single peak in fragment analysis were also sequenced with the same conditions described above.

All DNA samples were sequenced applying the BigDye Terminator v1.1 Cycle Sequencing Kit and 0.5 μg single-stranded DNA binding protein (Promega) on an Applied Biosystems 3130 Genetic Analyzer. Sequencing conditions were the following: Step 1: 96°C (2 min), step 2: 96°C (20 s), step 3: 56°C (20 s), step 4: 60°C (3 min), and step 5: 60°C (5 min). Steps 2–4 were repeated in 26 rounds. Data were analyzed with the software Chromas lite (Technelysium). All sequenced patients had given informed consent for research purposes in the DM2 field, as required by the declaration of Helsinki 2013.

TABLE 1 | Numbers of confirmed DM1 and DM2 positive patients in the period between 2004 and 2009 in the Würzburg lab.

	DM1			DM2		
Year	**Total**	**Positive**	**%**	**Total**	**Positive**	**%**
2004	170	110	64.7	270	115	42.6
2005	265	139	52.5	312	141	45.2
2006	233	94	40.3	314	103	32.8
2007	267	137	51.3	328	103	31.4
2008	221	102	46.2	396	106	26.8
2009	322	102	32.2	447	87	19.5

The total number represents the number of patients that was send to the lab with the clinical diagnosis of DM1 and/or DM2, respectively. Assignment to the suspected diagnoses was done by the treating physician. The number and percentage of positive patients represents the proportion of total patients in which the diagnosis DM1 or DM2, respectively, could be confirmed in our laboratory.

RESULTS

Prevalence of DM2

There are no well-documented prevalence data for DM2 except of the reports from Finland (11) and Rome Province in Italy (12), respectively. We are running one of the main diagnostic labs for neuromuscular diseases performing an estimated proportion of 75% of all tests for DM1/DM2 in Germany. From our own experience in the diagnostic lab, it is obvious that DM2 and DM1 contribute equally to the myotonic dystrophy phenotype as we detected about the same number of DM2 and DM1 patients by molecular genetic testing over many years (**Table 1**). Therefore, cumulative empiric evidence suggests that DM2 exhibits quite the same prevalence like DM1 in the German population. The submitters routinely send us both DM2 and DM1 samples, as is evident from the years shown in **Table 1**. In contrast to DM1, an indication for the test was generally given more liberally for DM2. Taking the different prevalences of DM1 from pre-molecular times (13) and making the assumption of a big phenotypical overlap due to a lack of diagnostic discriminability between DM1 and DM2 at that time, each of the two diseases should have about half of the previously stated prevalence. This would lead to an arithmetic prevalence of about 9 per 100,000 person-years or roughly 7,200 patients in Germany.

Statistical Analysis of Allele Length Frequencies

For this study, fragment analysis data were collected from 739 probands of presumed German origin in order to define the polymorphic spectrum of DM2 repeat tract lengths in the German population. **Figure 2** shows the frequency of alleles with different fragment lengths of DM2 negative probands. The combined repeat tract length was plotted against the allele frequency. In this set of samples, a unimodal distribution of 28 distinct allelic lengths is shown, ranging from 102 to 166 bp. The most frequent alleles in the German population consisted of 134, 138, 140, and 142 bp. Notably, while screening the normal alleles of DM2 positive patients in order to sequence the largest unexpanded alleles, we detected the longest allele at 168 bp, which is only 2 bp more than the longest allele (166 bp) found in DM2 negative patients (**Table 2**).

Expansion and Contraction of Probably Pathologic Alleles in Two Small Families

During our diagnostic procedures we observed the expansion of a $(CCTG)_{\sim 55}$ allele to a full expansion (family 1) and the contraction of a full expansion to a $(CCTG)_{\sim 30}$ allele (family 2, **Figure 3**). Both small expansion alleles are in the range of putative premutations (25–75 CCTGs) (4). The profiles of the repeat primed PCR (RP-PCR) analysis provide an approximate number of uninterrupted CCTGs in family 1. Phenotypic description of the two patients harboring the small expansion alleles were compatible with DM2: in family 1, symptoms of the mother with the small expansion fit the diagnosis DM2 including

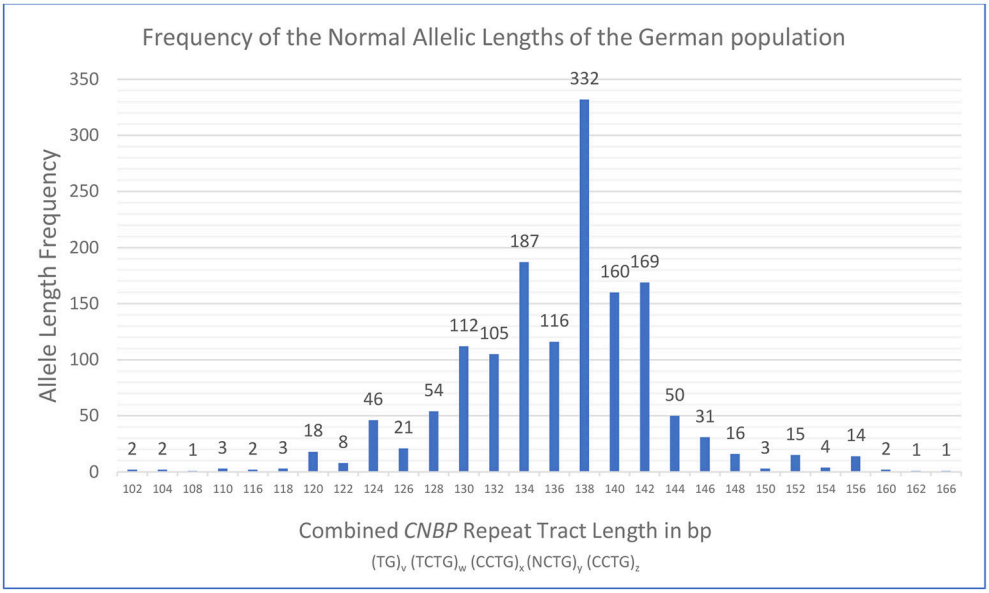

FIGURE 2 | Distribution and frequency of healthy range alleles of the *CNBP* repeat in the German population obtained from analysis of 1478 DM2 negative probands. Analysis revealed a range of the combined *CNBP* repeat tract lengths from 102 to 166 bp at the very extreme ends. Please note, that the longest healthy range allele observed in this study (168 bp) was found in combination with a pathological repeat expansion on the second allele in a DM2 positive patient and is therefore not part of the frequency plot of DM2 negative probands.

proximal weakness, myotonia and typical discharges in EMG. In family 2, the patient (daughter) with the contraction to about 30 CCTGs had a "mild myopathy" at the time of the test. Sixteen further premutation alleles ranging from about 26 to 55 CCTGs with an uninterrupted pattern in RP-PCR were observed in the last few years. The phenotype of the probands is in most cases not characteristic enough to make a clear diagnostic decision in favor of DM2.

Sequencing Analysis

The composition of the complex repeat motive of normal length alleles in the German population was analyzed by Sanger sequencing of DNAs from 34 DM2 patients with repeat expansion determined by routine diagnostic fragment analysis. Thirty one of the 34 DM2 positive patients were specifically selected because they possessed one of the three most frequent repeat lengths (136, 138, or 142 bp). The remaining three DNAs were derived from those three DM2 patients with a full expansion that revealed the three longest healthy range alleles found in our patient cohort (152, 156, and 168 bp). **Table 2** summarizes the compositions of specific alleles in DM2 positive patients. Moreover, eleven DM2 negative probands that appeared to be homozygous due to a single allele in fragment analysis were selected for sequencing to get an estimation of the real homozygosity frequency (data not shown).

In case of the 31 patients with the high abundance alleles (136, 138, or 142 bp), in all of the analyzed healthy range alleles of the *CNBP* repeat the CCTG tract was interrupted by three tetraplets $(NCTG)_3$: GCTG CCTG TCTG. The last CCTG repeat unit always comprised 7 CCTGs whereas the first CCTG unit before the interrupting $(NCTG)_3$ was found to vary between 5 and 6 CCTG tetraplets. Of the 31 sequenced alleles 26 revealed $(CCTG)_5(NCTG)_3(CCTG)_7$ (84%) while only 5 alleles (16%) had 6 leading CCTG tetraplets $[(CCTG)_6(NCTG)_3(CCTG)_7]$. In contrast to the highly stable CCTG tract, the $(TG)_v$ and $(TCTG)_w$ motifs at the beginning of the *CNBP* repeat stretch vary widely within the range of v = 17–25 $(TG)_v$ and w = 7–10 $(TCTG)_w$. The most frequent normal allele was identified to contain 9 TCTG tetraplets $[(TG)_v(TCTG)_9(CCTG)_5(NCTG)_3(CCTG)_7]$, with a frequency of 42% of the 31 analyzed alleles and a range of $(TG)_v$ of v = 19–23.

Composition of the two longest normal alleles (152 and 156 bp) was similar to each other: $(TG)_{22}$ $(TCTG)_w(CCTG)_8(NCTG)_5(CCTG)_6$. Specifically, in both alleles the interruption comprised five tetraplets: GCTG CCTG TCTG CCTG TCTG. The allele of 168 bp was found to be an exception with 23 uninterrupted CCTG tetraplets (**Table 2**).

Interestingly, only 2 of the 11 DM2-negative probands that appeared to be homozygous in the fragment analysis were indeed homozygous while the other 9 probands had repeat tracts with an identical length but a different motif composition where particularly TG and TCTG repeats varied widely. One of these true homozygous patients was homozygous for the allele $(TG)_{21}(TCTG)_9(CCTG)_5(NCTG)_3(CCTG)_7$ (138bp) while the other for $(TG)_{21}$ $(TCTG)_{10}(CCTG)_5(NCTG)_3(CCTG)_7$ (142bp). Another patient with homozygous allele lengths but heterozygous motif composition revealed a rare allele $(TG)_{21}$ $(TCTG)_7(CCTG)_6(NCTG)_3(CCTG)_9$ where the second CCTG motif had 9 instead of the constant number of 7 CCTGs (data not shown).

TABLE 2 | Composition of the combined repeat tract $[(TG)_v\ (TCTG)_w\ (CCTG)_x\ (NCTG)_y\ (CCTG)_z]$ of non-expanded, normal alleles in 34 DM2 positive patients determined by sequencing analysis.

Combined repeat size (bp)	$(TG)_v$	$(TCTG)_w$	$(CCTG)_x$	$(NCTG)_y$	$(CCTG)_z$
	v	w	x	y	z
134	21	8	5	3	7
	21	8	5	3	7
	21	8	5	3	7
	21	8	5	3	7
	19	9	5	3	7
	19	9	5	3	7
	19	9	5	3	7
	19	9	5	3	7
	17	10	5	3	7
	17	10	5	3	7
	21	7	6	3	7
	19	8	6	3	7
138	23	8	5	3	7
	23	8	5	3	7
	21	9	5	3	7
	21	9	5	3	7
	21	9	5	3	7
	21	9	5	3	7
	21	9	5	3	7
	21	9	5	3	7
	21	9	5	3	7
	21	9	5	3	7
	21	9	5	3	7
	19	10	5	3	7
	19	10	5	3	7
	19	10	5	3	7
	23	7	6	3	7
	21	8	6	3	7
142	23	9	5	3	7
	21	10	5	3	7
	21	10	5	3	7
	25	7	6	3	7
152	22	8	8	5	6
156	22	9	8	5	6
168	18	10	23	–	–

Indices v, w, x, y, and z denote the number of the specific repeat elements. While y and z were constant (y = 3, z = 7) throughout all 31 patients with repeat sizes of 134, 138, or 142 bp, x was found to be binary with x = 5 being about five times more frequent than x = 6. In contrast, v and w were highly variable with v ranging from 17 to 25 and w ranging from 7 to 10 in all 34 patients. The interruption of the two CCTG tracts by $(NCTG)_y$ was found to comprise three tetraplets in the high abundance alleles and five in two of the three large normal alleles (152 and 156 bp). The largest allele of 168 bp showed no interruption (y = 0) but 23 contiguous CCTGs.

DISCUSSION

Prevalence Data

The estimation of DM2 prevalence in Germany of about 9 in 100,000 people is equivalent to findings in the Italian population (12). For the German population it is quite clear from routine DM diagnostics that DM1 and DM2 have the same prevalence, but this varies in other countries. Moreover, there is increasing evidence from other reports that the DM2 prevalence in Europe is at least as high as that of DM1 or might be even higher (7, 11, 14). The reason of this relatively high prevalence in Northern Europe may be due to a founder effect (15) which was recently proposed as originating in the region of Slesia (3).

Distribution and Frequencies of Healthy Range *CNBP* Repeat Alleles

The distribution and the size range of short healthy range alleles of the *CNBP* repeat tract seem to be essentially similar among European populations. Comparing the German population's combined tract lengths $[(TG)_v(TCTG)_w(CCTG)_x(NCTG)_y(CCTG)_z]$ with those of the Slovak population underscores this hypothesis. The distribution of most healthy range alleles was found to be almost identical to the statistical results of the Slovak population (7): the range of normal alleles within 95 Slovak probands or 190 alleles was 118–156 bp with only one outlier of 184 bp with questionable clinical significance. In the bigger German cohort of 739 probands (or 1,478 alleles) most alleles were found to be situated in the same range between 120 and 156 bp. Due to the higher number of cases extreme ends of the distribution even extended from 102 to 166 bp including a few rare alleles.

The three most frequent allele lengths among the German population consisted of 138, 134, and 142 bp (most frequent to third-most frequent). Radvanszky et al. identified 138 bp as the most common tract length, which was found to be the most frequent in the German population, too. The second-most frequent allele length of 134 bp among the German population was in third place in the Slovak population, whereas the third-most frequent tract length of 142 bp in the German population was at second place among the Slovak allele lengths (7). Notably, abundance of the second and third most frequent alleles was very similar to each other in both our and the Slovak population.

Structural Composition of the Three Most Frequent Alleles in the German Population

The structure of the combined repeat tract is known to be highly polymorphic which is mainly attributed to the most polymorphic part, the $(TG)_v(TCTG)_w$ stretch. Accordingly, sequencing analysis of probands with only one apparently homozygous allele in the fragment analysis showed that fragment length alone does not predict homozygosity *per se* due to structural differences of the complex repeat. Indeed, a monomorphic structure was rarely observed (<20%). This is in accordance with other studies where heterozygosity was found to be roughly 90% in different European populations (4, 6, 7). Sequencing of the three most frequent alleles revealed only slight variation of the first CCTG stretch with x equaling either 5 or 6. The $(NCTG)_y(CCTG)_z$ part in contrast was fully stable in all 31 probands. The most common $(CCTG)_5(NCTG)_3(CCTG)_7$ allele accounted for 26 of the 31 alleles (84%) while only 5 (16%) belonged to the $(CCTG)_6(NCTG)_3(CCTG)_7$ allele. However, other rare alleles are present in the German population as

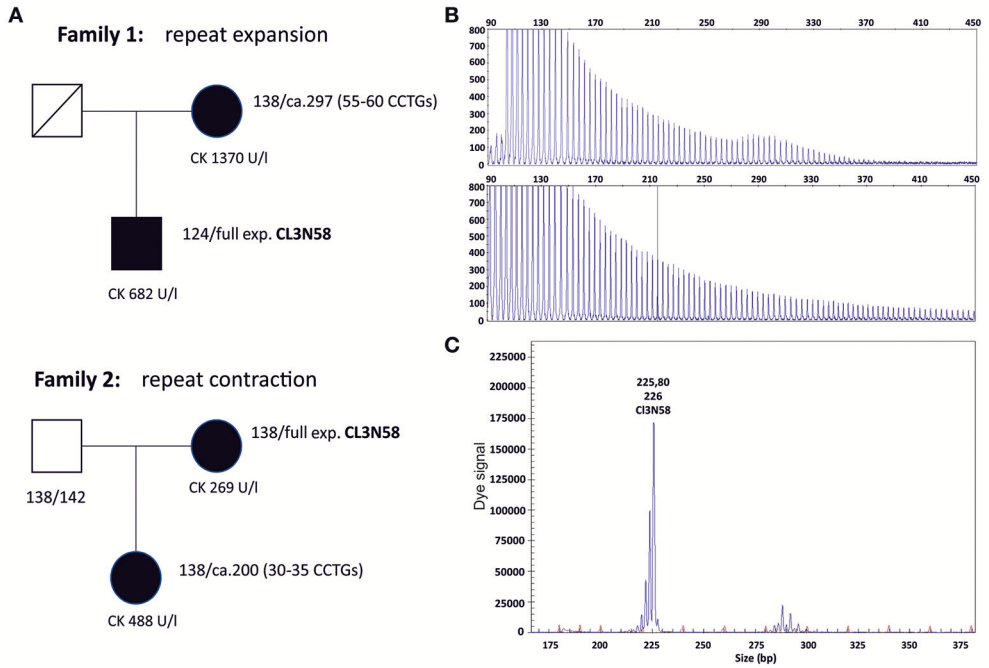

FIGURE 3 | (A) Pedigrees of two families with expansion and contraction events in meiosis. Family 1 shows a CCTG expansion from a premutation allele to a full expansion, family 2 a CCTG contraction from a full expansion to a premutation allele. Lengths of the combined repeat tract of both alleles are given in base pairs next to the patients. Using the repeat marker CL3N59 which is in linkage disequilibrium with the *CNBP* repeat marker (CL3N58), the contraction and expansion events could be linked to the respective expanded allele (data not shown). **(B)** Electropherograms of the repeat primed PCR (RP-PCR) of the affected members of family 1. The RP-PCR patterns are compatible with a pure CCTG stretch without interruptions by non CCTG tetraplets. **(C)** Fragment analysis of the contracted allele in family 2 (daughter). The premutation allele is represented by a wave of peaks. The fragment length (PCR product length) is ~290 bp, corresponding to a combined repeat tract of about 200 bp. All expansions were controlled by Southern blotting (data not shown).

observed in a "homozygous" but actually heterozygous DM2 negative proband, where a $(CCTG)_6(NCTG)_3(CCTG)_9$ allele with $z = 9$ instead of $z = 7$ was present. Our results correlate well with the observations in the Slovak population where 75.3% of probands contained the $(CCTG)_5(NCTG)_3(CCTG)_7$ and 17.4% the $(CCTG)_6(NCTG)_3(CCTG)_7$ allele (7) while only few other alleles could be detected. The interrupting motif $(NCTG)_y$ was always found to consist of the three consecutive tetraplets (CGTG)(CCTG)(TCTG) for the three most frequent alleles which has also been previously reported by Radvanszky et al. (7) and is considered to render the allele stable against expansion (2, 7).

Notably, the structural composition of 2 of the 3 longest analyzed normal alleles (152 and 156 bp) varied only in the amount of TCTG tetraplets. The CCTG interruption $(NCTG)_5$ consisted of an identical GCTG CCTG TCTG CCTG TCTG sequence in both alleles.

Conclusions From the German Cohort Analysis on Premutation Range, Repeat Instability, and Penetrance

It is suspected that frequent repeat tract lengths with interruptions are inherited stably in germline and somatically (2, 7). Accordingly, the CCTG tract of all 34 sequenced German alleles had an interruption of three $(CGTG)_1(CCTG)_1(TCTG)_1$ or five $(GCTG)_1(CCTG)_1(TCTG)_1(CCTG)_1(TCTG)_1$ tetraplets, respectively. Thus, the high polymorphic structure of the *CNBP* repeat is also recognizable from its interrupting tetraplets. However, uninterrupted alleles have previously been identified as being distributed over the whole size spectrum of 190 normal sized repeats (118–184 bp) and constituted 2.6% in the randomly selected Slovak individuals (7). These findings raise the question about where instability of uninterrupted alleles starts and whether a distinct instability threshold at a certain number of CCTGs can reliably be defined. Such a threshold would be expected at the upper end of the healthy repeat size range or beyond. The largest interrupted and therefore considerably stable repeat tract in Radvanszky's study encompassed 156 bp which is in accordance with our findings of a 152 and a 156 bp interrupted allele. Only few single alleles above that size could be identified in our analysis which supports a possible upper end of normal repeat tract size at around 156 bp.

The largest combined repeat tract length of presumed healthy range alleles in the German population was a 168 bp allele and was identified in a DM2 positive patient as second allele beside the expanded allele. Sequencing revealed that this rare allele has an uninterrupted stretch of 23 CCTGs but most probably does not contribute to the DM2 phenotype in the affected patient. Moreover, this CCTG stretch length is still

below an estimated general instability threshold of 100–200 bp or 25–55 repeats, respectively (16). For comparison, the largest identified allele in the randomly selected Slovak individuals was a single 184 bp allele that revealed even 30 uninterrupted CCTGs according to RP-PCR. However, this allele and the 23 CCTGs allele in our study might both already represent early instable or premutation alleles. Radvanszky et al. concluded from their findings in the Slovak population that the instability threshold most likely begins moderately at 30 uninterrupted CCTGs and further increases with the length of the CCTG stretch (7). In contrast, as observed in the members of family 2, contraction of an expanded allele to ∼30–35 uninterrupted CCTGs created a fully penetrant pathological allele of a size very close to healthy range alleles. The observation of an expansion and contraction from and to a fully penetrant "premutation" allele in the two families (**Figure 3**) shifts the potential pathological repeat range from 75 CCTGs to lower repeat values. It is worth to note that intergenerational contractions or *de novo* expansions of the *CNBP* repeat tract haven't been reported so far. The observation of transgenerational contraction events argues in favor of an unequal crossing over rather than strand slippage as a potential pathomechansim. It is not definitely clear whether these shorter uninterrupted CCTG stretches between 30 and 55 are fully penetrant, but at least in family 2 it is the case. We may face a bit of the situation in Huntington's disease: alleles with a lower number than 39 CAG repeats could be pathologic but are phenotypically not fully penetrant. Accordingly, we suggest that there may be an incomplete penetrance for premutation alleles in the range of ∼25–75 uninterrupted CCTGs.

CONCLUSION

Our observations in the German population lead to the assumption that alleles with a combined *CNBP* repeat tract length of around 156 bp can be considered to define the upper end of stable alleles as it represents the biggest interrupted normal allele from more than 700 probands and more than DM2 patients harboring an expansion. Premutation alleles without interruption could have originated from either an expansion event as well as from repeat contractions and can show full penetrance like in our two families 1 and 2. We therefore suspect that instability starts in the range of 25–30 uninterrupted CCTGs. According to findings for other repeat expansion diseases, like Huntington's disease, we suggest that the premutation range (25–75 CCTGs) shows incomplete penetrance which might depend on different parameters not known to date but could encompass specific changes in the repeat structure to patients' physical conditions or even environmental impacts. Collecting data from more patients with "small" DM2 expansions and known repeat tract structure as well as a follow up over time is necessary to estimate penetrance and should be a goal of future research to provide better genetic counseling for DM2 patients.

ETHICS STATEMENT

All subjects gave written informed consent in accordance with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

AM, TS, and WK data collection, data analysis, and data interpretation. AM, TS, WK, and SR drafting and critically revising the manuscript. BH-K, E-MH, and KS genetic testing.

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Myotonic Dystrophy—A Progeroid Disease?

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Myotonic dystrophies (DM) are slowly progressing multisystemic disorders caused by repeat expansions in the *DMPK* or *CNBP* genes. The multisystemic involvement in DM patients often reflects the appearance of accelerated aging. This is partly due to visible features such as cataracts, muscle weakness, and frontal baldness, but there are also less obvious features like cardiac arrhythmia, diabetes or hypogammaglobulinemia. These aging features suggest the hypothesis that DM could be a segmental progeroid disease. To identify the molecular cause of this characteristic appearance of accelerated aging we compare clinical features of DM to "typical" segmental progeroid disorders caused by mutations in DNA repair or nuclear envelope proteins. Furthermore, we characterize if this premature aging effect is also reflected on the cellular level in DM and investigate overlaps with "classical" progeroid disorders. To investigate the molecular similarities at the cellular level we use primary DM and control cell lines. This analysis reveals many similarities to progeroid syndromes linked to the nuclear envelope. Our comparison on both clinical and molecular levels argues for qualification of DM as a segmental progeroid disorder.

Keywords: myotonic dystrophy, segmental progeroid disorder, nuclear envelope, premature aging, DNA repair

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INTRODUCTION

Myotonic dystrophies (DM) are slowly progressing multisystemic disorders characterized by myotonia, muscle weakness, cataracts, and cardiac arrhythmia that can evolve into cardiomyopathy, insulin insensitivity and diabetes, testicular failure, and hypogammaglobulinemia (1, 2). The spectrum of DMs includes two types: type 1 (DM1) and type 2 (DM2) which are caused by mutations in two different genes. The age of onset of DMs ranges from congenital forms at birth to late onset at ∼70 years. Clinical symptoms cover muscular weakness, cataracts, balding, skin changes, and diabetes mellitus, often mirroring the appearance of accelerated aging. While the pathomechanism of DM has been shown to be a general splicing defect, it remains unclear what genes and splice variants yield particular pathologies in the affected tissues. Here, we provide a clinical description of aging symptoms in DM and compare this to "typical" progeroid disorders which mimic physiological aging and are caused by mutations in nuclear envelope (NE) proteins or DNA-repair proteins. Furthermore, we directly investigate some molecular hallmarks of aging in primary cell lines of DM patients.

MYOTONIC DYSTROPHY

Myotonic dystrophy (DM) can be caused by mutations in two genes: *DMPK* and *CNBP*. In both cases the disease is caused by an expansion of repeat elements within non-coding regions of the genes. Those repeats are transcribed and therefore contained within the pre-spliced mRNA. It is thought that RNA containing the expanded repeat forms hairpin structures and accumulates in foci in the nucleus. Several RNA-binding proteins are then recruited to these foci where they interact strongly with the mutant RNA. Among these proteins are MBNL proteins which are involved in alternative splicing (3). The accumulation of these proteins in the mRNA foci is thought to result in their depletion from the rest of the nucleus, resulting in turn in general mis-splicing and toxicity. This mis-splicing tends to revert the splicing pattern to comprise many embryonic splice variants. Phenotypically myotonic dystrophy is an autosomal dominant disease with predominant myotonia and muscle wasting. Furthermore eyes, heart, bone, skin, the endocrine system, gastrointestinal organs, and the central as well as the peripheral nervous system can be affected. The repeat length can vary between different tissues significantly (4). This complicates a prediction of the clinical development of patients as the repeat length is usually measured in DNA gained from blood.

DM1 causing mutations are an expansion of a CTG repeat in the 3' UTR of the *DMPK* gene (5). The general tendency is that the longer the expanded repeat the more severe the resulting phenotype is. Anticipation is commonly observed; the number of repeats typically increases in offspring over their parents so that the heritable disease tends to be of increasing generational severity. Up to 35 CTG triplets are considered normal, a repeat length between 35 and 49 is considered to be a premutation. Between 50 and ∼150 repeats have been observed in a mild expression of the phenotype and ∼100 to 1000 CTG repeats were identified in patients with classical DM. Repeats consisting of more than 1,000 CTG-triplets result in congenital DM, the most severe expression of the disease. The sexual inheritance also affects the severity of the disease: maternal inheritance results in more severe clinical features than paternal inheritance (6, 7).

Milder DM phenotypes can encompass cataracts, mild myotonia, or diabetes mellitus only, and the age of onset ranges between 20 and 70 years (8). Additional symptoms described in classical DM1 include distal muscle weakness, fatigue, cardiac conduction defects, neuropathy, endocrinopathies (on top of diabetes mellitus), and alopecia. Age of onset for the classical phenotype ranges between 10 and 30 years. In congenital DM1 affected children suffer severe and generalized weakness, hypotonia and respiratory problems after birth. One study further found that DM1 patients may have an increased risk of skin cancer (9).

DM2 mutations are located within intron 1 of the *CNBP* gene: more than 75 CCTG repeats have been described as disease causing (10). Unlike DM1 there is no described correlation between repeat length and disease severity in DM2. DM2 is considered a clinically more benign disorder than DM1 (11) and can be distinguished by a proximal muscular dystrophy and sparing of facial muscles (11), and the lack of a congenital form or the severe central nervous system involvement observed in DM1 (10, 12).

The fact that DM1 and DM2 are not clinically identical indicates that there are additional factors contributing to the disease pathomechanism besides the sequestration of splicing factors. In DM2 the repeat expansions tend to be longer than in DM1 so that one would expect DM2 to be more severe, but the opposite is the case: DM2 is clinically more benign. Thus it is possible that apart from the RNA toxicity the respective gene loci are contributing in different ways to the phenotype.

Due to its multisystemic involvement DM was suggested decades ago to be a segmental progeroid syndrome (13). Later it was also proposed as a model for premature muscle aging (14) and it is possible that in some mild cases DM might mimic sarcopenia (15, 16). Skin abnormalities frequently observed in DM1 and DM2 are also regarded as indicators for premature aging (17).

NUCLEAR ENVELOPE LINKED PROGEROID SYNDROMES

A group of progeroid disorders is caused by mutations in proteins of the nuclear envelope (NE) and also proteins involved in their processing. The NE is a double membrane system enclosing the genome in eukaryotic cells (18). Nuclear envelope transmembrane proteins (NETs) reside within the NE and it is underlaid by a meshwork of intermediate filament proteins, the nuclear lamina (19, 20). NE proteins lost or mutated in progeroid syndromes include lamin A and BAF. Apart from progeroid syndromes mutations in the lamin A encoding gene *LMNA* cause several tissue specific diseases (including muscular dystrophy, neuropathy and lipodystrophy). The progeroid syndromes caused by *LMNA* mutations encompass Hutchinson-Gilford progeria syndrome (HGPS), mandibuloacral dysplasia (MAD), Malouf syndrome, and several atypical progeroid syndromes that cannot be assigned clearly. Lamin A is an intermediate filament protein which undergoes post-translational processing for farnesylation. It has functions in mechanical stability, higher-order genome organization, chromatin regulation, transcription, DNA replication, and DNA repair (21). The major protein involved in its post-translational processing is the zinc metalloprotease STE24, encoded by the *ZMPSTE24* gene. Mutations in the *ZMPSTE24* gene cause mandibuloacral dysplasia (MAD) and restrictive dermopathy (RD). BAF (barrier to autointegration factor), a DNA binding protein, is encoded by the *BANF1* gene. Its functions include chromatin remodeling, gene expression, and DNA damage repair (22). It has been shown to interact with the LEM domain containing NETs emerin, MAN1 and Lap2β as well as with lamin A (23–25).

Hutchinson-Gilford progeria syndrome (HGPS) is most commonly caused by the *de novo* heterozygous *LMNA* mutation c.1824C>T; p.G608G which activates a cryptic splice site and causes the deletion of 50 amino acids. This deletion includes the cleavage site necessary for maturation of lamin A by post-translational processing (26, 27). Affected individuals appear

healthy at birth, but develop a progeroid phenotype within 1–2 years. This comprises a short stature, low body weight, early loss of hair, loss of subcutaneous fat, localized scleroderma-like skin conditions, osteolysis, and facial features resembling aging (small face and jaw, prominent eyes, pinched nose, thin lips, and protruding ears). In most cases cardiovascular problems are the reason for death in the second decade of life (28, 29).

Mandibuloacral dysplasia (MAD) can be caused by recessive mutations in *LMNA* [MADA, (30)] or *ZMPSTE24* [MADB, (31)]. While the *LMNA* mutations tend to be homozygous or compound heterozygous missense mutations, *ZMPSTE24* mutations resulting in MADB tend to be a combination of missense and nonsense mutations (32). Patients are characterized by postnatal growth retardation, craniofacial anomalies with mandibular hypoplasia, skeletal malformations, osteolysis of distal phalanges, and clavicles, skin changes such as atrophy, and speckled hyperpigmentation, insulin resistance, and diabetes, and lipodystrophy which appears to be partial in MADA and generalized in MADB (32).

Restrictive dermopathy (RD) is caused by homozygous or compound heterozygous nonsense *ZMPSTE24* mutations resulting in a loss of the protein (33). The term RD describes a rare, lethal, genodermatosis. Affected children die before birth or within the first week of life. Clinical features include tightly adherent thin skin, prominent vessels, characteristic facial features ("O" shaped mouth), generalized joint contractures, dysplasia of clavicles and respiratory insufficiency (32, 34).

Malouf syndrome is caused by heterozygous *LMNA* mutations within the N-terminal parts of lamin A. In 2003 mutations were identified in patients originally described as suffering from Werner syndrome, but with no mutation in the *RECQL2* gene and therefore named atypical Werner syndrome (35)– though if the clinical phenotypes were actually matching Werner syndrome was not absolutely clear (36, 37). Later work (38) noted the phenotypic similarity to patients described by Malouf et al. (39). Described clinical findings include hypergonadotropic hypogonadism, cardiomyopathy, blepharoptosis, mild mental retardation, prominent nasal bones, scleroderma-like skin, and lipodystrophy (35, 38, 39).

In addition to these progeroid disorders there are several cases of so called atypical progeroid syndromes caused by *LMNA* mutations. These cases are often linked to a specific mutation and show overlaps between well described *LMNA* or *ZMPSTE24* linked diseases. Therefore it's not possible to assign them clearly to a syndrome (40–44).

Nestor-Guillermo progeria syndrome (NGPS) is caused by recessive mutations in the *BANF1* gene (45). Patients start to develop a failure to thrive at age of 2, the skin becomes dry and atrophic and they develop a generalized lipoatrophy, osteoporosis, and osteolysis.

Amongst these several NE-linked progeroid syndromes, the age of onset and life expectancy vary, but similarities include skin abnormalities (scleroderma-like, atrophy or speckled hyperpigmentation), osteolysis/osteoporosis, loss of hair, cardiac involvement, insulin resistance, typical facies, and in some cases muscular weakness. No noteworthy increased risk of cancer amongst these disorders has been reported (**Table 1**).

DNA Repair Linked Progeroid Syndromes

Another group of progeroid diseases are caused by mutations in DNA-repair proteins. Those encompass mutations in RecQ protein-like helicases (RECQL) and nuclear excision repair (NER) proteins. RecQ helicases play major roles in genome maintenance and stability (46). Mutations in genes encoding members of this protein family are causative for the premature aging disorders Werner syndrome and Bloom syndrome. NER proteins repair single stranded DNA damage—particular UV-induced DNA damage. Progeroid syndromes caused by mutations in NER protein encoding genes include Cockayne syndrome, Xeroderma pigmentosum, and Trichothiodystrophy.

Werner syndrome is caused by recessive mutations in the RECQL2 protein encoding *WRN* gene that result in a loss of protein by creating new stop codons or cause frameshifts resulting in a premature stop codon (47). RECQL2 is involved in DNA double-strand break repair where it regulates the pathway choice between classical and alternative non-homologous end joining (48) and relocalizes from the nucleolus to other nuclear regions upon DNA damage (49). It is suggested to be involved in telomere replication (50). Werner syndrome patients have scleroderma-like skin changes, cataracts, osteoporosis, arteriosclerosis, diabetes mellitus, cancer, characteristic "birdlike" facies, and can have alopecia (51).

Mutations in the *BLM* gene, encoding RECQL3, cause Bloom syndrome. The inheritance is recessive, and mutations result in a loss of protein or loss of function (52, 53). RECQL3 is involved in DNA replication and repair, where it acts in several steps during homologous recombination during DNA double-strand break repair (54). Patients present with pre- and postnatal growth deficiency, UV-sensitivity, hypo-, and hyperpigmented skin, and predisposition to malignancy (55).

Cockayne syndrome (CS) is distinguished into type A [CSA, caused by recessive *ERCC8* mutations (56)] and type B [CSB, caused by recessive *ERCC6* mutations (57)]. CSA patients show a progeroid appearance with slow growth and development, skin photosensitivity, thin and dry hair, pigmentary retinopathy, sensorineural hearing loss and dental caries (58). CSB patients are characterized by failure to thrive, severe mental retardation, congenital cataracts, loss of adipose tissue, joint contractures, distinctive face with small, deep-set eyes, and prominent nasal bridge, kyphosis, sensorineural hearing loss, and cachectic dwarfism (59).

Xeroderma pigmentosum (XP) is a rare autosomal recessive disorder with patients showing acute photosensitivity and a predisposition to skin cancer on sun-exposed areas of the body (60). XP is caused by mutations in the *XPA*, *ERCC3*, *XPC*, *ERCC2*, *DDB2*, *ERCC4*, *ERCC5,* and *POLH* genes. Another NER protein associated disease is Trichothiodystrophy (TTD). Patients display a wide variety of clinical features which includes cutaneous, neurologic and growth abnormalities as well as intellectual/developmental disabilities, ocular abnormalities and decreased fertility (61). Causative mutations have been described in the *ERCC3*, *GTF2H5*, *MPLKIP*, *GTF2E2*, *ERCC2,* and *RNF113A* genes.

TABLE 1 | Overview comparing myotonic dystrophies with selected nuclear envelope- and DNA repair- linked progeroid syndromes.

Disease	Type	Gene	Age of onset	Age of death	Skin	Bones	Eyes	Muscle /Fat	Developmental delay	Neuro-degeneration	Diabetes mellitus	Hair	Cancer
Myotonic Dystrophy type 1 (DM1)	Mild	*DMPK*	20–70 yrs	60 yrs–normal	dysplastic nevi, alopecia, xerosis and seborrheic dermatitis		cataracts	myotonia	no		diabetes mellitus	alopecia	possibly increased risk
	Classic		10–30 yrs	48–55 yrs	dysplastic nevi, alopecia, xerosis and seborrheic dermatitis		cataracts	myotonia, muscular dystrophy	no	axonal peripheral neuropathy, CNS involvment	diabetes mellitus	alopecia	possibly increased risk
	Congenital		Birth to 10 yrs	Neonatal / 45 yrs	dysplastic nevi, alopecia, xerosis and seborrheic dermatitis		cataracts	muscular dystrophy	yes	yes	diabetes mellitus		possibly increased risk
Myotonic Dystrophy type 2 (DM2)		*CNBP*	3rd decade	Sudden death due to cardiac involvement possible	dysplastic nevi, alopecia, xerosis and seborrheic dermatitis		cataracts	myotonia, muscular dystrophy	no	in a few cases	diabetes mellitus	rarely alopecia	possibly increased risk
Hutchinson-Gilford progeria syndrome (HGPS)		*LMNA*	1–2 yrs	14 yrs	scleroderma-like	osteolysis	no	partial lipodystrophy	yes	no	diabetes mellitus	loss	no increased risk
Mandibuloacral dysplasia (MAD)	MADA	*LMNA*	4-5 yrs	normal life expectancy	skin atrophy, calcinosis	osteolysis	no	partial lipodystrophy	yes	no	diabetes mellitus	alopecia	no increased risk
	MADB	*ZMPSTE24*	2 yrs		skin atrophy	osteolysis	no	generalized lipodystrophy	yes	no	diabetes mellitus	alopecia	no increased risk
Restrictive dermopathy (RD)		*ZMPSTE24*	Antenatal, Neonatal	mean age of 13.5 years	hyperkeratosis		hypertelorism, entropion	no	yes			absent/spare eyebrows, -lashes, lanugo	no increased risk
Malouf syndrome		*LMNA*	Infancy, neonatal	18-26 yrs	no	osteoporosis	ptosis	lipodystrophy	no	mental retardation (some patients)	no	no	no increased risk
Nestor-Guillermo progeria syndrome (NGPS)		*BANF1*	2 yrs	Third decade of life	dry, atrophic	osteoporosis, osteolysis	propotosis	generalized lipoatrophy	yes	no	no	loss	no increased risk
Werner syndrome		*WRN*	Median age 13 yrs	Median age of 54	scleroderma-like	osteoporosis	cataracts	Muscle atrophy	yes	brain atrophy in 40%	diabetes mellitus	loss, premature greying	increased risk
Bloom syndrome		*BLM*	birth	Median age 27	photosensitivity, pigmentation abnormalities	no	no	no	yes	mild retardation, learning disability (some patients)	non-insulin-dependent diabetes mellitus	hypertrichosis	increased risk
Cockayne syndrome (CS)	CSA	*ERCC8*	1 yr	12 yrs	photosensivity, wrikeled and premature aged		cataracts, pigmentary retinopathy	denervation myopathy	yes	intellectual disability	diabetes mellitus	thin, dry and premature greying	no increased risk
	CSB	*ERCC6*	birth	7 yrs						severe mental retardation			

In general these DNA repair linked progeroid disorders exhibit a frequent involvement of the skin (scleroderma-like, hyperpigmentation, increased photosensitivity), osteoporosis, and cataracts occur, and there is also frequently neuronal involvement. In addition this group of disorders tends to have an increased risk of cancer (**Table 1**).

DM—ACCELERATED AGING AT THE MOLECULAR LEVEL?

Aging related defects can be observed at the cellular level in cells from patients with premature-aging disorders. Cellular hallmarks of aging include senescence, telomere attrition, genomic instability, mitochondrial dysfunction, and loss of proteostasis (62). There are observations of premature senescence in DM cells — cells obtained from distal muscle of congenital DM1 patients show a reduced proliferative capacity and an increased rate of telomere shortening. The reduced proliferative capacity observed in these cells was thought to be caused by a p16 dependent premature senescence (63, 64). DM2 myoblasts have also been shown to reach premature senescence, but in a p16 independent manner (65). Congenital DM1, but not DM2 myoblasts show differentiation defects (66, 67). There are also changes in epigenetic marks in both DM1 and DM2 patient cells, suggesting possible changes to genome organization: DM2 myoblasts exhibit heterochromatin accumulation (68) and the DM1 locus is methylated to varying degrees across the expanded repeats (69) and especially in congenital samples (70).

Another aging-associated feature is mitochondrial dysfunction. It is proposed that mitochondrial free radicals cause oxidative damage which is a driving force in cellular aging (71). Mutations in mitochondrial DNA (mtDNA) can lead to premature aging (72, 73). Increased mtDNA deletions have been reported in DM (74). Furthermore mis-regulation of the mitochondrial protein CoQ10 has been described in DM in generell (75) and EFTu, HSP60, GRP75 as well as Dienoyl-CoA-Isomerase specifically in DM2 (76). Another aging-linked feature is the loss of proteostasis. Proteostasis has been shown to collapse during aging (77) and there are indications that this occurs in DM1 and DM2: CTG repeat expressing mice activate the ubiquitin-proteasome pathway (78) and altered protein degradation has been shown in DM2 myotubes (76).

OVERLAPS WITH TYPICAL PROGEROID DISORDERS

DNA repair failure is certainly involved in the expansion of the repeats in both DM1 and DM2; this is potentially caused by slippage of DNA polymerase (79, 80). NER has also been shown to promote repeat expansion (81) and a polymorphism the MSH3 mismatch repair gene has been associated with somatic repeat instability (82). However, there are also reports indicating NE abnormalities in DM. In DM1 derived fibroblasts an altered localization of lamin A, lamin B1, and the NET emerin have been described (83). This altered localization includes distribution to invaginations of the NE, also known as nucleoplasmic reticuli (84). Knockdown of the zinc metalloprotease STE24, which is mutated in the progeroid syndromes MADB and RD and is a major player in the processing of prelamin A to mature lamin A, results in an enrichment of nucleoplasmic reticuli (85).

MATERIALS AND METHODS

Patient and Controls

Primary human myoblast were obtained from the Muscle Tissue Culture Collection (MTCC) at the Friedrich-Baur-Institute (Department of Neurology, Ludwig-Maximilians-University, Munich, Germany). All control and patient materials were obtained with written informed consent of the donor. Ethical approval for this study was obtained from the ethical review committee at the Ludwig-Maximilians-University, Munich, Germany (reference 45-14). Repeat length was diagnosed on DNA extracted from blood. Age and sex of patients and controls are listed in **Table 2**.

TABLE 2 | Primary myoblast cell lines used and characterization.

Sample	Sex	Age at biopsy in years	Repeat length	Muscle of origin	Positive for Ki-67 staining in %	Positive for desmin staining in %	Used for immune-fluorescence	Used for western blot
Ctrl-1	♂	43	–	M. biceps brachii	22.3	96.2	yes	yes
Ctrl-2	♀	36	–	M. biceps brachii	42.0	68.4	yes	no
Ctrl-3	♀	49	–	M. vastus lateralis	27.0	95.2	yes	yes
DM1-1	♂	38	200	unknown	n.d.	n.d.	yes	no
DM1-2	♂	34	240–430	M. deltoideus	16.6	70.1	yes	yes
DM1-3	♀	33	300–500	unknown	26.6	83.3	yes	yes
DM1-4	♂	27	400–600	unknown	n.d.	100	yes	no
DM1-5	♀	29	800–1500	unknown	37.9	50.3	yes	yes
DM2-1	♂	31	n.d.	unknown	57.6	48.0	yes	yes
DM2-2	♀	32	n.d.	M. vastus lateralis	43.0	86.0	yes	yes
DM2-3	♂	41	n.d.	M. rectus femoris	43.4	45.0	yes	yes
DM2-4	♀	37	n.d.	M. biceps brachii	n.d.	n.d.	yes	no
DM2-5	♂	35	n.d.	M. biceps brachii	20.0	94.4	yes	no

Tissue Culture

Myoblasts were grown in tissue culture using skeletal muscle cell growth medium (PeloBiotec, Munich, Germany). Cells were kept from reaching confluency to avoid differentiation. Passage numbers were matched for controls and patient cells for the respective experiments, throughout all experiments passage numbers 8 to 10 have been used. For differentiation DMEM containing 5% HS, was used. Myotubes were differentiated for 7 days. Cells were grown at 37°C in a 5% CO2 incubator.

Immunohistochemistry

Myoblasts were fixed with methanol (−20°C). Following primary antibodies were used for staining: Ki-67 (Thermo Scientific, RM-9106-S0), emerin 5D10 and lamin A/C 4A7 (both provided by Glenn E. Morris). All secondary antibodies were Alexafluor

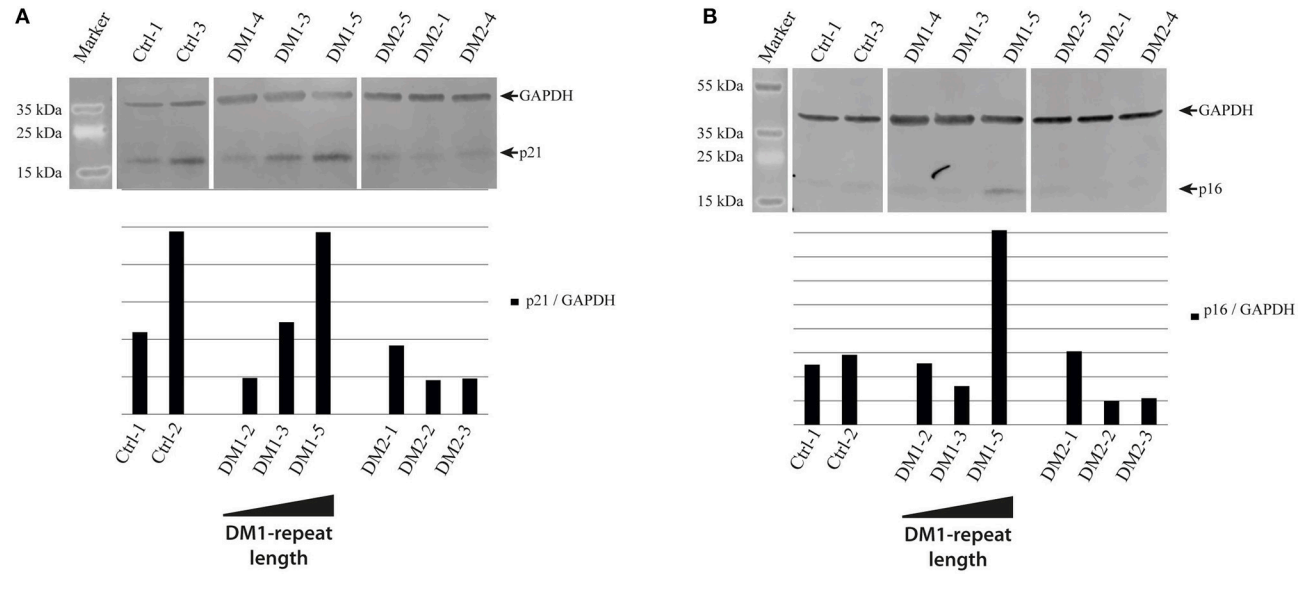

FIGURE 1 | Cell cycle regulatory proteins in myotonic dystrophy. Western Blot and quantification of primary control, DM1 and DM2 myoblasts for cell cycle regulatory proteins p21 **(A)** and p16 **(B).** DM1 samples are ordered according their diagnosed repeat length from left (small repeat) to right (long repeat).

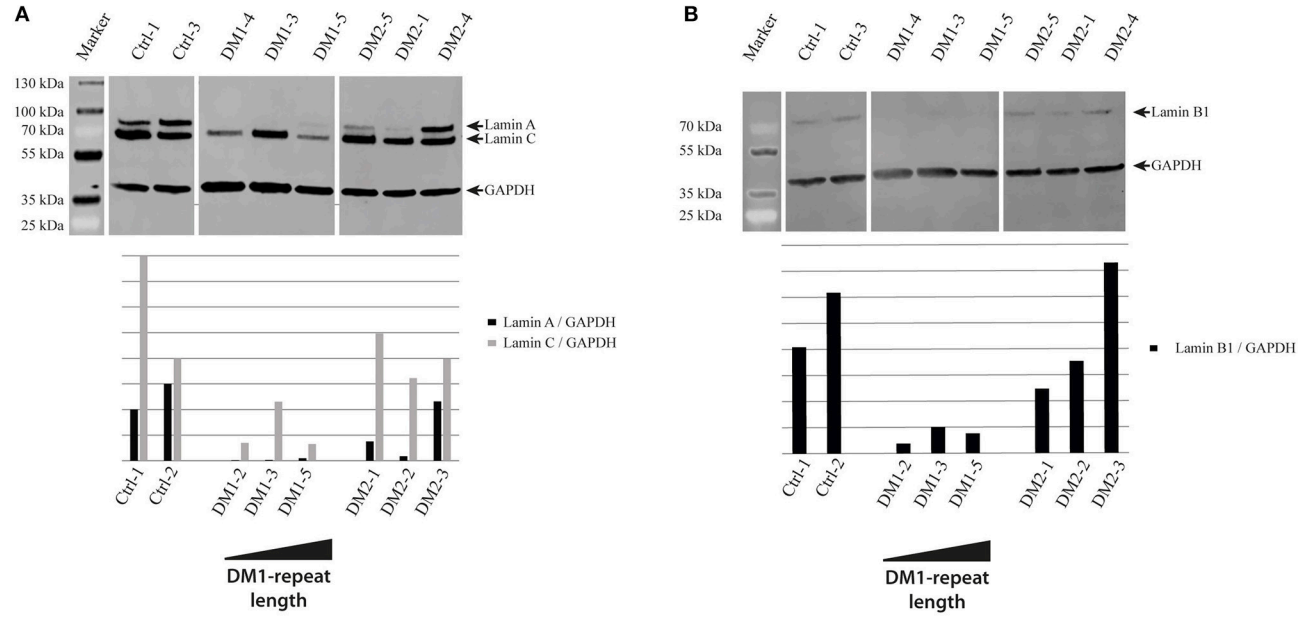

FIGURE 2 | Lamina proteins in myotonic dystrophy. Western Blot and quantification of primary control, DM1 and DM2 myoblasts for lamin A and lamin C **(A)** and lamin B1 **(B).** DM1 samples are ordered according their diagnosed repeat length from left (small repeat) to right (long repeat).

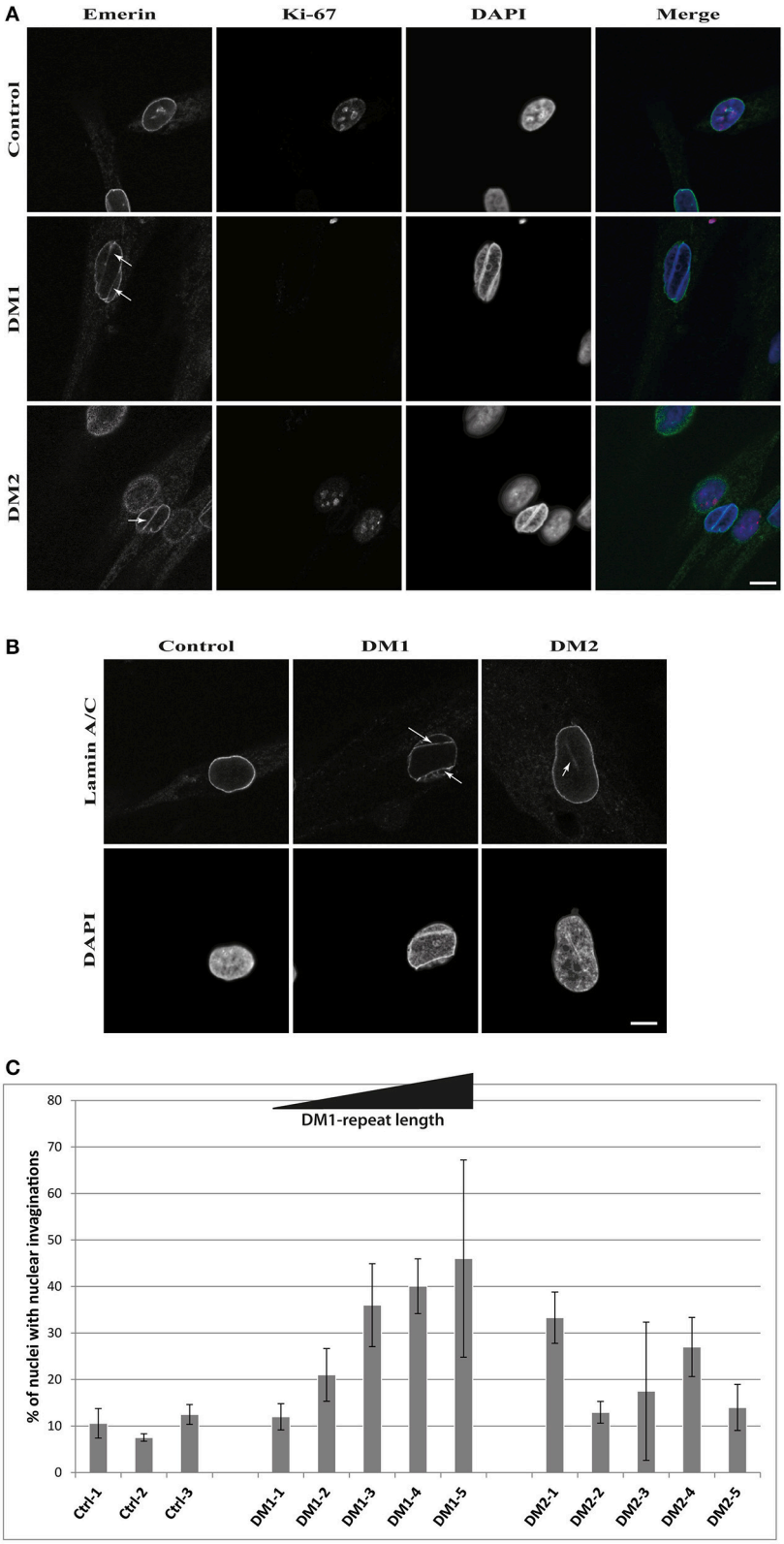

FIGURE 3 | Nuclear envelope invaginations in myotonic dystrophy. Immunofluorescence staining of primary control, DM1 and DM2 myoblasts for **(A)** emerin and Ki-67 showing nuclear envelope invagination in DM1 and DM2 myoblasts, **(B)** confirmation of nuclear envelope invaginations by lamin A/C staining and **(C)** quantification of these structures in DM and control cell lines—standard deviation is shown. White arrows indicate invaginations of the nuclear envelope. Scale bar 10 μm.

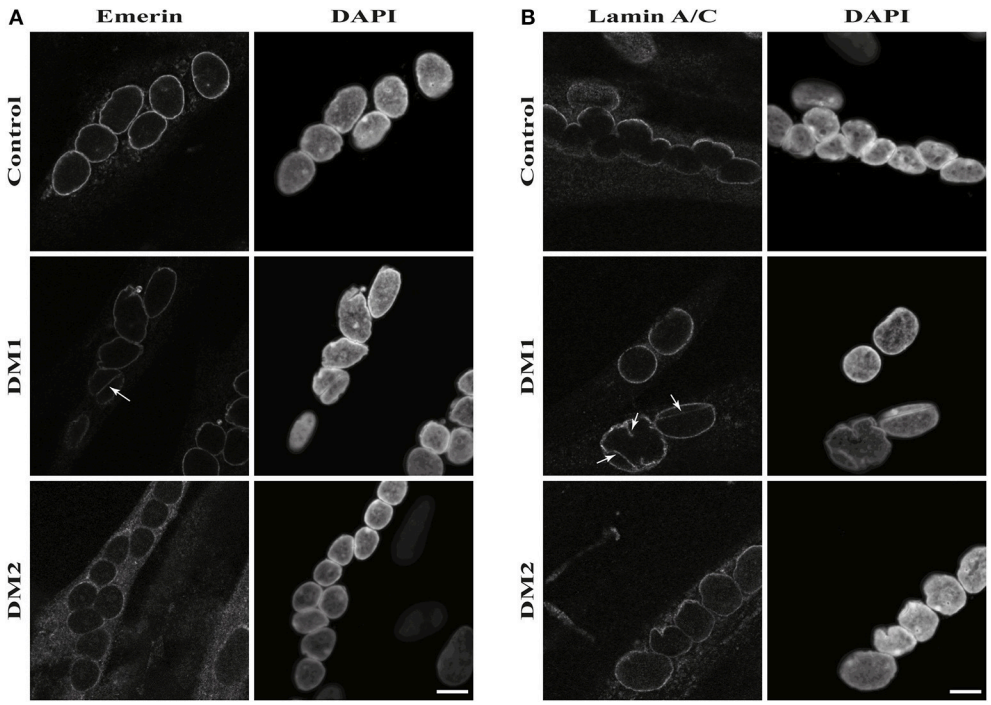

FIGURE 4 | Nuclear envelope invaginations in myotonic dystrophy and control myotubes. Immunofluorescence staining of primary control, DM1 and DM2 myotubes for **(A)** emerin and **(B)** lamin A/C showing nuclear envelope invagination in DM1 myotubes. White arrows indicate invaginations of the nuclear envelope. Scale bar 10 μm.

conjugated and generated in donkey with minimal species cross-reactivity. DNA was visualized with DAPI (4,6-diamidino-2 phenylindole, dihydrochloride).

Microscopy and Image Analysis

All images were obtained using an Olympus FluoView FV1000/BX 61microscope equipped with a 1.42 NA 60x objective and 3x zoom magnification. Image analysis was performed using ImageJ software. For quantification of nuclear invaginations at least 100 nuclei were counted for each measurement with the Olympus FluoView FV1000/BX 61 confocal microscope using the Z-drive to investigate the whole nucleus. For each sample at least two or three (depending on the fitness of each individual cell line) biological replicates were analyzed.

Western Blotting

Whole protein extracts were generated from myoblast cell cultures using an ultrasonic sonicator with a MS73 tip (Bandelin Sonopuls). The proteins were separated by SDS gel electrophoresis using 4–15% TGX gels (BioRad #456–8087). Western blotting was performed using the Trans-Blot® TurboTM system (BioRad). Proteins were transferred to low fluorescent PVDF membranes (part of Trans-Blot® TurboTM RTA Transfer Kit #170-4274). Membranes were blocked with 5% BSA or 5% skim milk in 1xTBS/0, 1% Tween® 20. Following primary antibodies were used: lamin A/C 4A7 (provided by Glenn E. Morris), lamin B1 (D4Q4Z, Cell Signaling) p16INK4A (ab108349, Abcam), p21 (Cell Signaling #2947). For quantification mouse antiGAPDH (Milipore #MAB374) or rabbit antiGAPDH (Cell Signaling GAPDH (D16H11) XP #5174) were used. As secondary antibodies we used donkey anti-mouse IRDye 680RD, donkey anti-mouse IRDye 800CW, donkey anti-rabbit IRDye 680RD and donkey anti-rabbit IRDye 800 CW. All western blot images were obtained using a Licor FC. Quantification was done using the Licor ImageStudio Software. Western blots were repeated at least two times to confirm the results.

RESULTS

As cellular senescence is a hallmark of aging we decided to initially analyze cell cycle proteins linked to senescence in our primary human myoblast cell lines. First, we used Western blot analysis to quantify the expression of p21 and p16 (**Figure 1**). P21 is an inhibitor of the cell cycle (86), which fails to be up-regulated in DM2 myoblasts during differentiation (87). Western blot analysis of our myoblast cell lines shows only small changes in p21 expression (**Figure 1A**), however this may be within the observed expression level variations in primary myoblasts. P16 is a tumor suppressor protein that has previously been shown to be mis-regulated in congenital DM1 samples (63). There were no changes in p16 expression except in the DM1 cell line with the longest repeat where the expression was elevated. The next step was to quantify lamin B1, another senescence associated biomarker (88) which has not been investigated in DM before. While we do not see lamin B1 changes in DM2 myoblasts, it was down-regulated in all DM1 myoblasts tested (**Figure 2B**).

The results of the lamin B1 quantification led us to further investigate effects on NE proteins. For this we quantified the expression of the other lamin subtypes A and C, finding that lamin A is strongly down-regulated in DM1 (**Figure 2A**). Immunofluorescence staining for lamin A/C shows an increased number of nuclei with invaginations in DM1 and DM2 myoblasts (**Figure 3B**). This has been confirmed and quantified by staining with the NET emerin (**Figures 3A,C**). For DM1, primary patient cell lines with longer CTG repeats show a greater percentage of nuclei with nuclear invaginations (**Figure 3C**). Co-staining with the proliferation marker Ki-67 revealed that all cells with nuclear invaginations are negative for Ki-67 and hence senescent. Staining of differentiated myotubes with lamin A/C and emerin shows the presence of nuclear invaginations in DM1 myotubes (**Figures 4A,B**).

DISCUSSION

The differences in cell cycle control described in DM1 and DM2 myoblasts gave rise to the idea that there are different pathomechanisms in each DM type. While for DM2 a down-regulation of the cell cycle inhibitor p21 has been shown (87), there is a reported mis-regulation of p16 in congenital DM1 (63). We can confirm both effects in our DM patient myoblasts, although the effects observed by us are not very strong and the mis-regulation of p16 in DM1 seems to be restricted to longer repeats (**Figure 1**).

Looking for a better senescence marker we quantified lamin B1, a protein of the NE that has been shown to be down-regulated in HGPS (89), cellular senescence (90) and normal aging (91). The down-regulation of lamin B1 occurs specifically in DM1 myoblasts and is independent of the repeat length (**Figure 2B**). This indicates that it could be a more relevant marker for DM1 in general than p16. Furthermore, the strong down-regulation of lamin A (**Figure 2A**) confirms that there are strong effects on the expression of nuclear lamina proteins in DM1. The composition of the nuclear lamina is important to achieve its multiple functions including mechanical stability, chromatin organization, transcriptional regulation, and response to oxidative stress (92, 93)–and the down-regulation of the lamins A and B1 is potentially having an effect on all these functions thus contributing to the phenotype.

Further evidence of NE involvement in DM comes from the enrichment of nuclear envelope invaginations in DM myoblasts (**Figures 3**, **4**). DM myoblasts show more nuclei with these structures, with the strongest effects being observed in DM1. Moreover, all nuclei with invaginations have exited the cell cycle. In contrast to the lamin B1 levels, there appears to be a correlation of repeat length in DM1 and the percentage of nuclei positive for NE invaginations. The longer the diagnosed DM1 repeat the more myoblast nuclei in primary cell lines gained from those patients have invaginations of the nuclear envelope. This could be another overlap to NE-linked progeroid syndromes: knockdown of ZMPSTE24 also results in an enrichment of NE invaginations (85) and the loss of ZMPSTE24 in human

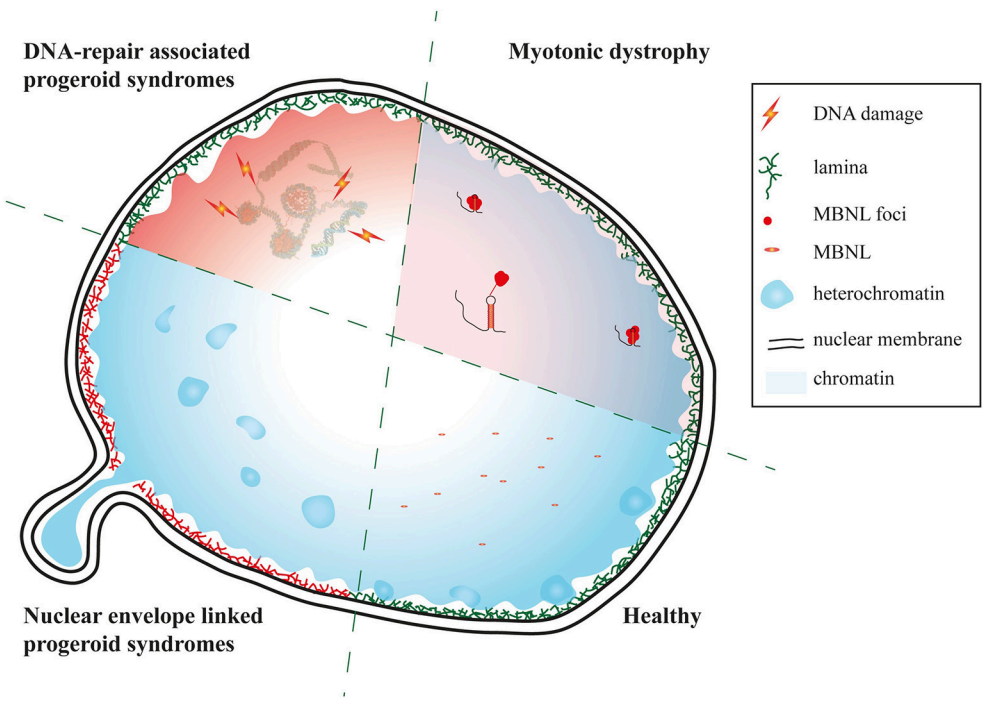

FIGURE 5 | Myotonic dystrophy as a facet of progeroid syndromes? Schematic of affected nuclear regions and pathways in DM and nuclear envelope as well as DNA repair linked progeroid syndromes.

results in RD, the most severe NE-linked progeroid syndrome (32). A possible explanation of how this contributes to the disease pathology is that mis-regulation of lamina proteins results in NE aberrations which force the cell to exit the cell cycle and enter senescence. Consequently, this might deplete the pool of myoblasts during muscle regeneration and contribute to the muscular dystrophy in DM via failed regeneration.

Taken together our results suggest that there is on both the clinical and molecular level clear evidence that DM reflects facets of segmental progeroid disorders (**Figure 5**).

CONCLUSION

DMs qualify by clinical phenotypes as well as molecular features as segmental progeroid syndromes. In DM1 the composition of the NE is altered and there is an enrichment of nuclear invaginations likely contributing to the phenotype. As several NE-linked syndromes have muscle involvement, this further suggests the possibility of an overlap between NE-linked progseroid syndromes and DM1.

AUTHOR CONTRIBUTIONS

PM and BS contributed to the conception and design of the experiments. PM wrote the manuscript, SH and SL performed the experiments.

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REVIEW
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Of Mice and Men: Advances in the Understanding of Neuromuscular Aspects of Myotonic Dystrophy

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Intensive effort has been directed toward the modeling of myotonic dystrophy (DM) in mice, in order to reproduce human disease and to provide useful tools to investigate molecular and cellular pathogenesis and test efficient therapies. Mouse models have contributed to dissect the multifaceted impact of the DM mutation in various tissues, cell types and in a pleiotropy of pathways, through the expression of toxic RNA transcripts. Changes in alternative splicing, transcription, translation, intracellular RNA localization, polyadenylation, miRNA metabolism and phosphorylation of disease intermediates have been described in different tissues. Some of these events have been directly associated with specific disease symptoms in the skeletal muscle and heart of mice, offering the molecular explanation for individual disease phenotypes. In the central nervous system (CNS), however, the situation is more complex. We still do not know how the molecular abnormalities described translate into CNS dysfunction, nor do we know if the correction of individual molecular events will provide significant therapeutic benefits. The variability in model design and phenotypes described so far requires a thorough and critical analysis. In this review we discuss the recent contributions of mouse models to the understanding of neuromuscular aspects of disease, therapy development, and we provide a reflective assessment of our current limitations and pressing questions that remain unanswered.

Keywords: myotonic dystrophy, mouse, trinucleotide DNA repeat, skeletal muscle, cardiac muscle, central nervous system, brain, RNA toxicity

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INTRODUCTION

Animal models offer experimental tools to investigate the causes and mechanisms of disease, when the access to human samples is limited. The remarkable progresses in genetic engineering allowed the introduction of human mutations in the mouse genome, to reproduce molecular, cellular and physiological disease manifestations. The resulting phenotypes provide insight to confirm starting hypotheses, reveal novel pathogenic mechanisms and evaluate new therapies. Myotonic dystrophy (DM) illustrates the cardinal contribution of mouse models to the systematic dissection of a complex disease mechanism, from genetic mutation to the design of clinical trials.

DM is the most common form of adult muscular dystrophy, characterized by pleiotropic symptoms, which are highly variable in their nature and severity (1). Major muscular features include myotonia, muscle weakness, atrophy and smooth muscle dysfunction. Cardiac conduction

defects and arrhythmias are associated with cardiomyopathy and may lead to sudden death (1). Brain involvement is illustrated by predominant structural abnormalities of the white matter, cognitive impairment (such as executive dysfunction, visuospatial deficits and abnormal social cognition), behavioral changes (such as apathy and social avoidance) and excessive daytime sleepiness (2). Other peripheral disease manifestations include insulin resistance, iridescent posterior subcapsular cataracts, and gastrointestinal complications (such as constipation/diarrhea) (1).

Two different autosomal dominant mutations in two unrelated genes cause DM and define two genetically distinct forms of the condition. DM type 1 (DM1) is caused by the expansion of a CTG trinucleotide repeat in the 3′-untranslated region (UTR) of the DM protein kinase (*DMPK*) gene (3). The DM type 2 (DM2) mutation consists in the expansion of an intronic CCTG tetranucleotide in the CCHC-type zinc finger nucleic acid binding protein (*CNBP*) gene (4). Although genetically distinct, DM1 and DM2 share a toxic RNA gain of function mechanism. In both conditions, expanded CUG/CCUG transcripts accumulate in the cell nucleus to form RNA aggregates or RNA *foci* (4–6), which perturb the function of RNA-binding proteins and a number of downstream events (7). Although clinically similar, disease symptoms are usually milder in DM2 than in DM1 (1, 8).

In a scenario where the expansion of simple non-coding DNA repeats has a broad deleterious impact on multiple tissues and physiological processes the generation of mouse models that faithfully reproduce the disease presents unique challenges. In order to be clinically relevant mouse models must have *construct, face* and *predictive* value (9). In other words, relevant mouse models must recapitulate the genetics and molecular pathogenesis (construct value); they must mimic clinical human features, both molecularly and physiologically (face value); and they must provide a platform to determine the effectiveness of new therapeutic interventions on a clinical population (predictive value). However, mouse models rarely, if ever, completely recapitulate all aspects of human disease. This is particularly applicable to DM, given the clinical variability of the disease, the involvement of multiple tissues and the complexity of the underlying molecular pathways. Even with this caveat, mouse models, alone or in combination, have been instrumental to understand fundamental molecular pathomechanisms (10). Importantly, they have allowed molecular and cellular analyses at various developmental stages, as well as in cell types and tissues that are not easily accessible in humans. We have previously reviewed the contribution of mouse models to decipher the grounds of RNA toxicity and to evaluate promising preclinical assays (10), but there is little doubt that mouse models have continued to provide in-depth understanding of DM disease mechanisms over the last years.

Here we discuss how recent mouse data refined our understanding of RNA toxicity and unfolded numerous roles and pathogenic implications of the RNA-binding proteins dysregulated in DM. We review other emerging disease intermediates and dysregulated signaling pathways recently uncovered. Pre-clinical therapeutic developments are discussed in light of their contribution to reinforce fundamental aspects of disease pathogenesis. We focus primarily on the neuromuscular aspects of the disease to establish correlations between mouse data and human pathology. We point out some contradictory findings between mouse models to illustrate the challenges, complexity and variability of DM disease pathogenesis.

FROM DNA REPEATS TO TOXIC RNA TRANSCRIPTS

The toxicity of RNA repeats was unequivocally demonstrated in HSA^{LR} transgenic mice, through the insertion of an expanded CTG sequence in the 3′UTR of an unrelated gene: the human actin, alpha 1 (*ACTA1*) gene. The expression of CUG-containing *ACTA1* transcripts in mouse skeletal muscle generated genuine myotonia and histological signs of myopathy (11). The elimination of the expanded transcripts by antisense oligonucleotides reduced myotonia in these mice (12), confirming the toxicity of CUG RNA repeats.

The absence of muscle weakness in the HSA^{LR} mouse line that expressed the highest transgene levels and showed pronounced muscle histopathology was intriguing and suggested the dissociation between the toxicity of RNA foci and the etiology of muscle weakness (11), an hypothesis that persisted for some years. However, the later analysis of a second HSA^{LR} line, which also expressed high levels of the transgene and showed myotonia, revealed reduced grip strength (13). Contrary to the initial reports, these findings corroborate the view that the expression of toxic RNA repeats is sufficient to trigger muscle weakness. CUG RNA toxicity was further demonstrated and confirmed in other mouse lines, listed in **Table 1.**

The ubiquitous expression of expanded *DMPK* transcripts from the human DM1 locus resulted in multisystemic phenotypes in DMSXL mice carrying more than 1000 CTG repeats. These phenotypes include reduced muscle strength, lower motor performances, peripheral neuropathy, respiratory impairment, abnormal cognition and behavior, and cardiac conduction defects (26–29). Similarly, the inducible expression of a large, interrupted CTG repeat flanked by the 3′UTR of the *DMPK* gene produced cardiac, muscular and neurological phenotypes in EpA960 mice (16, 30, 31). Surprisingly, high expression of short $(CTG)_5$ repeats within the *DMPK* 3′UTR was pathogenic in DM5 mice, causing DM1-like myotonia and cardiac conduction defects (17). Hence, the expression of many copies of a short CUG repeat may have functional outcomes that are comparable to the expression of a few copies of large CUG RNA repeats. In other words, the toxicity of repetitive RNA is two-fold: it is determined not only by the sequence length but also by the abundance of the repeat transcripts in the cell. While HSA^{LR}, DMSXL and EpA960 animals accumulate foci, nuclear RNA aggregates were not detected in DM5 mice, raising the possibility that submicroscopic RNA foci can cause disease, or that soluble CUG RNA is also pathogenic (32). The DM1 molecular hallmarks reported in the main poly-CUG mouse

TABLE 1 | Summary of transgene design and expression in the DM mouse models most extensively studied.

Models of toxic RNA expression (poly-CUG models)

Mouse model	(CTG)n	Flanking sequence	Promoter	Tissue expression	References
HSA^{LR}	~250	Human skeletal actin 3′UTR	Human *ACTA1*	Skeletal muscle	(11)
DMSXL	>1,000	Human DMPK locus	Human *DMPK*	Ubiquitous	(14, 15)
EpA960	960	Human DMPK 3′UTR	CMV	Inducible (ubiquitous or tissue-specific)	(16)
DM5/DM200	5/200	Tet-responsive, human *DMPK* promoter	Human *DMPK*	Inducible (ubiquitous or tissue specific)	(17)

Models of altered RNA-binding proteins

Mouse model	Mutation/construct	Tissue expression	References
Mbnl1 KO	Constitutive deletion of *Mbnl1* exon 3	Ubiquitous	(18)
Mbnl2 KO	Constitutive deletion of *Mbnl2* exon 3	Ubiquitous	(19)
Mbnl3 KO	Constitutive deletion of *Mbnl3* exon 3	Ubiquitous	(20)
Mbnl1/Mbnl2 DKO	Constitutive deletion of *Mbnl1* exon 3 Constitutive or conditional deletion of *Mbnl2*	Ubiquitous deletion of *Mbnl1*. Ubiquitous or tissue-specific deletion of *Mbnl2*	(21)
Mbnl1/Mbnl3 DKO	Constitutive deletion of *Mbnl1* exon 3 Constitutive deletion of *Mbnl1* exon 2		(22)
Mbnl1/Mbnl2/Mbnl3 TKO	Constitutive deletion of *Mbnl1* exon 3 Conditional deletion of *Mbnl2* and *Mbnl3*	Ubiquitous deletion of *Mbnl1*. Tissue-specific deletion of *Mbnl2* and *Mbnl2*	(23)
TRECUGBP1	Human CELF1 sequence downstream of Tet-responsive CMV promoter	Inducible (ubiquitous or tissue-specific)	(24)
TRECUGBP2	Human CELF2 sequence downstream of Tet-responsive CMV promoter	Inducible (ubiquitous or tissue-specific)	(25)

TABLE 2 | Molecular hallmarks of RNA toxicity in the mouse models expressing CUG RNA repeats.

Models of toxic RNA expression (poly-CUG models)

Mouse model	RNA foci	MBNL co-localization	CELF1 upregulation	Missplicing	References
HSA^{LR}	Skeletal muscle	MBNL1	Skeletal muscle	Severe in skeletal muscle	(11, 13, 33)
DMSXL	Multiple tissues	MBNL1 MBNL2	Brain (and CELF2) Trend in heart	Mild, age-dependent in multiple tissues	(26, 27)
EpA960	Skeletal muscle; Heart; CNS	MBNL1 MBNL2	Skeletal muscle Heart Brain	Severe in skeletal muscle and heart. Mild in brain	(16, 30, 31)
DM5/DM200	Absent	Not detected	Skeletal muscle. Normal levels in heart	Mild in skeletal muscle. Absent in heart	(17)

models are summarized in **Table 2.** No poly-CCUG DM2 mouse model has been fully characterized yet.

RNA foci are dynamic ribonucleoproteic structures that disrupt important RNA-binding proteins (**Figure 1**). Members of the MBNL (muscleblind-like) family of splicing factors are sequestered and partially inactivated by the RNA foci in DM1 and DM2 (34, 35), while CELF (CUGBP Elav-like family) proteins are abnormally upregulated, at least in DM1 (16, 36, 37). MBNL sequestration, CELF upregulation and missplicing have been detected to different extents in mouse models expressing poly-CUG RNA transcripts (**Table 2**). MBNL and CELF proteins bind independently to RNA targets and functionally compete to regulate their downstream processing (25). The two protein families comprise key regulators of developmental splicing

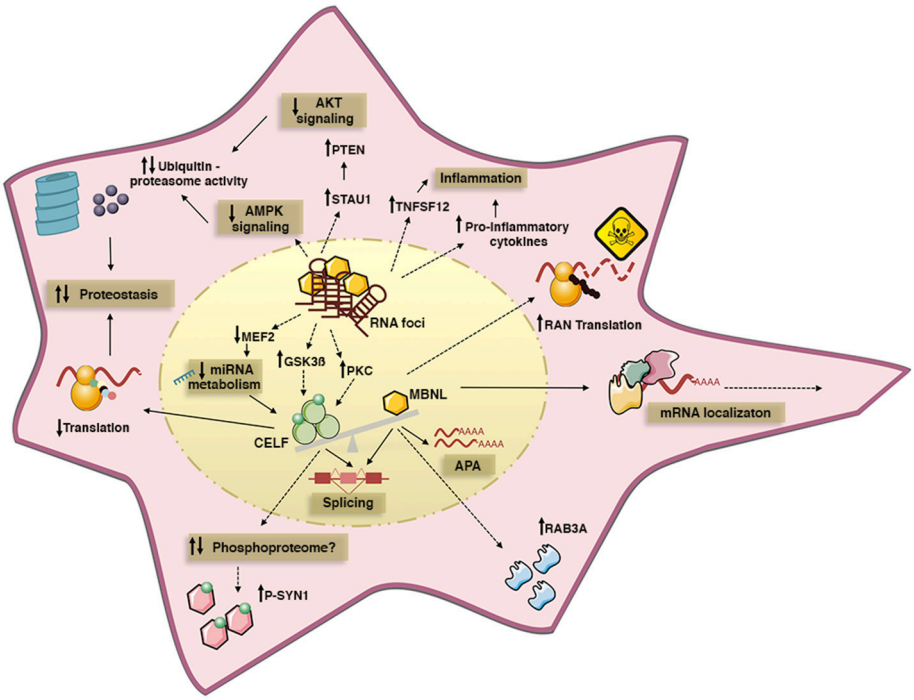

FIGURE 1 | Summary of some of the cell pathways and signaling cascades dysregulated by toxic RNA repeats in DM cells. The expression of toxic RNA transcripts sequesters MBNL proteins into nuclear RNA foci, and upregulates CELF proteins. Different mechanisms may account for CELF upregulation, such as altered PKC and GSK3ß kinase activity, or changes in miRNA levels due to altered MEF2 transcription program. MBNL inactivation and CELF gain-of-function cause pathogenic missplicing. Functional MBNL inactivation alone disrupts alternative polyadenylation and intracellular localization of mRNA targets; it is also believed to dysregulate protein expression, independently of splicing, and to promote RAN translation of toxic peptides. In turn, CELF1 upregulation affects translation efficacy and it may affect the phosphorylation of a subset of proteins through unidentified mechanisms. Protein homeostasis is also perturbed by the downregulation of AKT and AMPK signaling pathways, which likely promotes protein catabolism by increased ubiquitin-proteasome activity, hence contributing to muscle atrophy and weakness. Finally, the increased expression of pro-inflammatory cytokines suggests ongoing inflammation in DM. Solid lines represent well defined disease mechanisms, while dashed lines represent circumstantial data with poorly defined mechanistic links.

transitions. The combined MBNL sequestration and CELF upregulation results in the pathogenic expression of fetal isoforms in adult DM tissues (7). In other words, DM spliceopathy does not produce "unusual" splicing isoforms; instead, it is associated with the expression of normal splicing products that are not well-suited to adult tissue function, leading to the onset of typical disease manifestations. In this context myotonia is the consequence of the abnormal splicing of the CLCN1 chloride channel (38, 39), while insulin resistance is most likely associated with the missplicing of the insulin receptor (38, 40). It is important to note the significant overlap between the splicing abnormalities in DM and other muscular dystrophies (41). The similarities depict a scenario in which splicing dysregulation in DM is not only a primary disease process, but also a secondary event caused by general tissue degeneration.

In addition to the canonical sense transcripts, both DM1 and DM2 loci produce antisense transcripts, a feature shared with many microsatellite repeat loci and that has been suggested to regulate local gene expression (42). The CTG expansion interferes with the relative levels of sense and antisense RNA in DM1 patients (43) and in transgenic mice carrying the human DM1 locus (44). The pathogenic impact of these changes on local gene expression and disease mechanisms requires further studies in experimental models.

THE MULTIFACETED ROLE OF MBNL PROTEINS IN DM PATHOGENESIS

Humans and mice (as well as most vertebrates) express three *MBNL* genes (*MBNL1*, *MBNL2*, and *MBNL3*) (45). Endogenous MBNL1 and MBNL2 co-localize with CUG and CCUG RNA foci in DM1 and DM2 cells, respectively (4, 46–48), whereas MBNL3 protein was not detected in adult tissues (48).

The three *MBNL* paralogs show differences in spatial distribution in adult mouse tissues. *Mbnl1* and *Mbnl2* transcripts are ubiquitously expressed, but *Mbnl1* RNA levels are higher in heart, whereas *Mbnl2* is more homogenously distributed (49). The steady-state levels of MBNL2 protein, however, are low in adult skeletal muscle (19). *Mbnl3* transcript levels are very low in adult mice (49). Differences in protein distribution extend to cell types: the analysis of primary mouse cultures revealed higher relative levels of MBNL1 in astrocytes, while MBNL2 was more abundant in primary neurons (50).

The involvement of MBNL proteins in DM was tested in knockout lines generated either through the deletion of *Mbnl* genes alone, or the combined inactivation of multiple *Mbnl* genes (**Table 1**). These mice revealed some degree of functional specialization between individual members of the MBNL family and clarified their roles in disease molecular pathogenesis.

Functional Specialization of MBNL Proteins: Insight From Single Knockout Lines

Direct evidence of detrimental MBNL sequestration was provided by the generation of *Mbnl1* KO mice. *Mbnl1* inactivation impacted primarily the skeletal muscle and caused pronounced myotonia, but it also resulted in DM1-like subcapsular cataracts, lack of motivation and apathy in knockout mice (18, 51). The impact on cardiac function was less obvious and dependent on the genetic background of *Mbnl1* KO mice: cardiac conduction defects were more pronounced on a homogenous 129/Sv background (52), relative to a mixed 129/Sv x C57BL6 background (21). The reasons behind strain-specific cardiac differences between the homogenous and the mixed background have not yet been resolved, but the comparison between these two lines may provide unique insight into the modifiers of disease severity. It is important to note that DM is a highly variable condition, and that variability in disease manifestations may be explained by a complex interplay between genetic modifiers and environmental factors. The backcrossing of different mouse models onto different genetic backgrounds may facilitate the identification of relevant genetic modifiers of disease.

Although reproducing critical muscular and cardiac phenotypes, *Mbnl1* KO mice did not develop prominent muscle weakness/wasting or marked cognitive deficits, aside from decreased motivation (51). Additional MBNL members may therefore serve as key disease intermediates. Indeed, the inactivation of *Mbnl2* yielded mild muscle pathology, but marked CNS phenotypes, suggesting a tissue-specific impact of *Mbnl* gene inactivation. Neurological phenotypes of *Mbnl2* KO include sleep disturbance, defective spatial memory, abnormal synaptic plasticity and seizure susceptibility (19).

The deleterious effect of *Mbnl1* inactivation on muscle physiology was accompanied by splicing defects that are more severe in skeletal muscle and in heart than in the CNS (18, 52, 53), and it related to the role of MBNL1 in the control of fetal-to-adult splicing transitions in muscle (18, 33). Similarly, MBNL2 appears to serve a similar function in the CNS (19). As a result of this regional specialization, *Mbnl1* KO mice express embryonic splicing isoforms predominantly in the muscle (18, 33), while *Mbnl2* KO mice exhibit embryonic splicing profiles mainly in the CNS (19). Still, we cannot exclude other significant roles of MBNL1 in the CNS independent of splicing: MBNL1 controls the steady-state levels of RAB3A, and possibly other synaptic proteins (27, 54); and it also determines the length of neuronal dendrites and axons (31) (**Figure 2**).

The inactivation of *Mbnl3* yielded intriguing results: despite low *Mbnl3* expression in adult muscle, *Mbnl3* KO mice exhibited reduced grip strength and age-dependent decline in skeletal muscle regeneration (20). Other age-associated phenotypes were described in an independent *Mbnl3* KO line, such as glucose intolerance, cardiac deficits and subcapsular cataracts (55). MBNL3 loss of function may therefore contribute to the accelerated aging suggested in DM (56). Interestingly, the phenotypes of *Mbnl3* KO mice are not accompanied by significant changes in alternative splicing (20, 55). Together with the primary localization of MBNL3 in the cytoplasm (23), these findings predict roles of MBNL proteins other than splicing regulation.

Combined Inactivation of MBNL Proteins

Despite the significant phenotypes of *Mbnl1* and *Mbnl2* single gene knockout lines, they do not model the full disease spectrum, possibly due to compensatory mechanisms of the remaining *Mbnl* genes (21). In *Mbnl1* KO mice, *Mbnl2* expression is upregulated and MBNL2 protein binds to target transcripts that are normally regulated by MBNL1 (21). In order to recreate a situation that resembles more closely the human disease, in which the three MBNL paralogs are sequestered by toxic RNA foci (35), compound knockout mice were generated (**Table 1**). While *Mbnl1/Mbnl2* double knockout (DKO) mice were embryonic lethal, the inactivation of one *Mbnl2* copy in a *Mbnl1* KO background was sufficient to exacerbate myotonia and trigger muscle weakness, loss of mature neuromuscular junctions and cardiac conduction defects, which were absent in single *Mbnl1* KO mice (21, 57). The aggravated phenotypes were accompanied by an increasing severity in spliceopathy and significant changes in alternative polyadenylation (APA) (58). The molecular analysis of *Mbnl1/Mbnl2* DKO mice was instrumental to reveal the role of MBNL proteins in the regulation of APA: MBNL proteins and the APA machinery compete to bind to APA sites of a subset of transcripts, in a mechanism that regulates the processing and length of the 3′ end of target transcripts, with subsequent implications for their stability and localization (**Figure 1**). Similar to DM splicing abnormalities, the sequestration and functional inactivation of MBNL proteins by toxic RNA results in the persistence of fetal APA profiles in adult muscle and brain of DM1 and DM2 patients (58, 59). The direct contribution of individual APA defects to specific symptoms is unclear (60), but the mouse models available offer unique tools to address this question, not only in DM but also in other conditions in which MBNL proteins are sequestered by toxic RNA repeats.

Dual depletion of *Mbnl1* and *Mbnl3* also enhanced myotonia, muscle weakness and myopathy in skeletal muscle (22). However, in contrast to *Mbnl1/Mbnl2* DKO, increased myotonia was not associated with a greater extent of splicing dysregulation in *Mbnl1/Mbnl3* DKO mice. Instead, enhanced myotonia was the result of the synergy between *Clcn1* missplicing caused by *Mbnl1* deletion alone, and defective CLCN1 translation, caused by combined inactivation of MBNL1 and MBNL3 proteins (22).

More recently, conditional triple knockout (TKO) mice were generated by muscle-specific deletion *Mbnl2* and *Mbnl3* on an

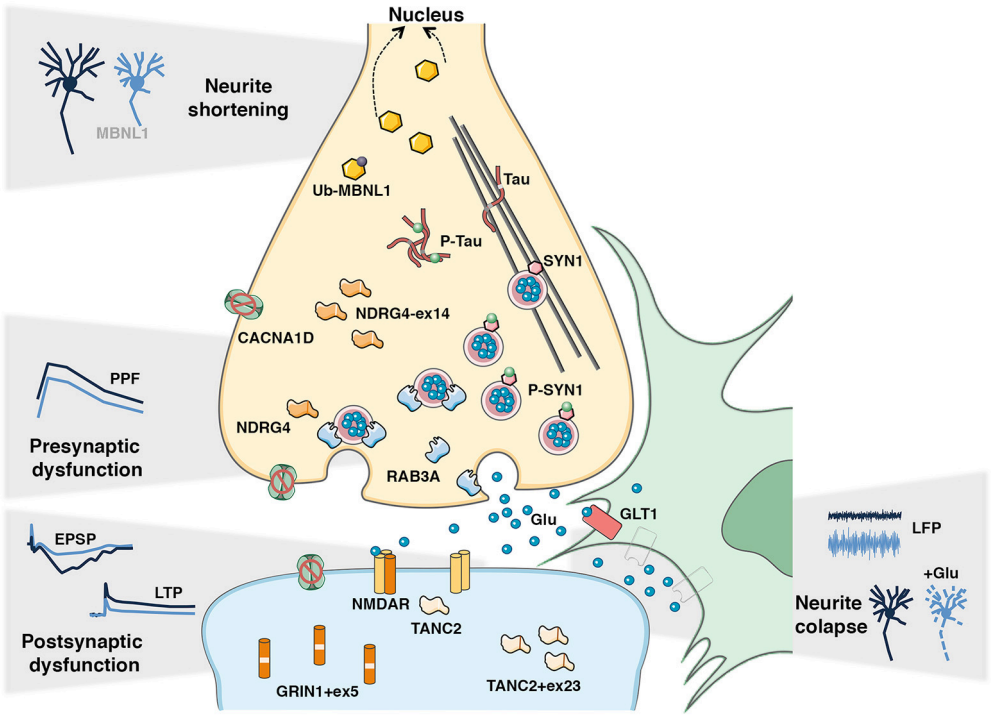

FIGURE 2 | Candidate disease intermediates of DM synaptic dysfunction and learning deficits. The dysregulated disease mechanisms in the CNS of DM1 mouse models appear to involve both pre- and postsynaptic events, which lead to global synaptic dysfunction and consequent cognitive and memory deficits. In the pre-synaptic compartment the hyperphosphorylation of SYN1 and upregulation of RAB3A, together with the missplicing of *Mapt/Tau*, *Ndrg4*, and *Cacna1d* may contribute to impaired short-term synaptic plasticity, notably through decreased paired-pulse facilitation (PPF) detected in DMSXL mice. In the postsynaptic counterpart, the missplicing of *Grin1*, *Tanc2*, and *Cacna1d* may disrupt the functioning of the voltage-gated NMDA receptor, and consequently NMDAR-mediated mechanisms of long-term potentiation (LTP) detected in *Mbnl2* KO and EpA960 mice. Reduced GLT1 levels in neighboring astrocytes likely result in neuronal hyperexcitability, demonstrated by increased local field potentiation (LFP) in DMSXL mice, and it can ultimately lead to neuronal damage and neurite collapse in the presence of excessive glutamate. The mislocalization of MBNL1 into the nucleus following abnormal de-ubiquitination decreases neuritogenesis and affects neuronal morphology in EpA960 mice. Together these events likely mediate defective synaptic transmission and abnormal brain connectivity behind DM cognitive and behavioral changes.

Mbnl1 knockout background (**Table 1**). *Mbnl1/Mbnl2/Mbnl3* TKOs present high neonatal mortality, growth defects, respiratory distress, muscle weakness and wasting in association with pronounced splicing and gene expression defects (23). Interestingly, the total spliceopathy in muscle was only modestly increased in *Mbnl1/Mbnl2/Mbnl3* TKO mice, relative to *Mbnl1/Mbnl2* DKOs, supporting the view that congenital spliceopathy is primarily due to compound loss of MBNL1 and MBNL2, and further pointing to MBNL3 functions, other than splicing regulation. Hence, the congenital form of the disease seems to require combined inactivation of the three *Mbnl* paralogs from an early developmental stage. DMSXL mice, which also show growth retardation from birth, and DM1 individuals express both sense and anti-sense *DMPK* transcripts from embryonic and fetal stages (44), confirming that the toxic RNA mechanisms behind congenital cases could operate early on during development.

Overall the generation and characterization of single and compound *Mbnl* KO mouse models, demonstrated that the simultaneous sequestration of various MBNL proteins is instrumental for the development of clinical manifestations of DM. Given the sparse availability and technical difficulties of working with human DM tissue, constitutive and conditional *Mbnl* KO mice grant the possibility to investigate abnormal RNA processing in different tissues, cell types and developmental stages.

Insight From MBNL Replacement Strategies

MBNL1 loss of function accounts more than 80% of missplicing events and nearly 70% of expression defects in the skeletal muscle of HSA^{LR} mice (61, 62), strongly anticipating the benefits of therapeutic gene replacement. Overexpression of MBNL1 through viral infection or genetic manipulation ameliorated myotonia and splicing abnormalities in the tibialis anterior of HSA^{LR} mice, however it was insufficient to fully correct muscle histopathology (63, 64). While further confirming the role of *Mbnl1* loss of function in the onset of myotonia, these findings also hint at the involvement of additional disease intermediates in muscle pathology. It is conceivable that other MBNL proteins might be necessary to fully reverse muscle phenotypes. Prior to the further development of MBNL

replacement strategies, it is important to evaluate to which extent MBNL proteins are interchangeable and capable to functionally replace each other in muscle and in other tissues of DM1 mouse models expressing expanded CUG transcripts. Alternatively, the incomplete rescuing of muscle physiology in HSA^{LR} mice by MBNL1 overexpression points to the involvement of other families of disease intermediates alongside MBNL proteins.

Cytoplasmic Roles of MBNL: RNA Trafficking and Proteotoxicity

MBNL proteins are also present in the cytoplasm (23, 48), where they likely regulate mRNA stability (61, 62, 65), as well as the intracellular localization of mRNA transcripts through binding to the 3′UTR of their targets (66, 67). The role of MBNL proteins in mRNA trafficking might be particularly relevant in highly polarized brain cells, such as neurons. Altered MBNL activity or intracellular localization in DM1 could be detrimental for correct transport of mRNAs toward specialized cell compartments (such as axons, dendrites and synapses), which would subsequently affect local translation and ultimately cell function.

In further support of a cytoplasmic function of MBNL proteins, the expression of CUG RNA in the forebrain of EpA960 mice (**Table 1**) affects MBNL1 ubiquitination and distribution between the nucleus and the cytoplasm (**Figure 2**), prior to the shortening of neuronal dendrites and axons (31, 68). Morphological impairments occurred in the absence of missplicing, suggesting a contribution of cytoplasmic MBNL1 to disease process.

MBNL proteins have been recently proposed to act as guardians against proteotoxicity. CUG and CCUG RNA generate toxic peptides through non-conventional repeat-associated non-ATG (RAN) translation (69, 70). The combination of bidirectional transcription with RAN translation of expanded repeats produces multiple toxic species, which co-localize with markers of apoptosis, supporting a role in disease pathology (71). Interestingly, RNA accumulation seems to co-exist and exacerbate RAN translation in the same cell, in a mechanism mediated by MBNL proteins: MBNL sequestration and inactivation by nuclear RNA foci promotes RAN translation in DM1 and DM2 cell models (70, 72). RAN products have been reported in DMSXL mice (69). Future mouse studies are required to elucidate the relationship between RNA foci, MBNL protein and RAN translation, and the pathogenicity of RAN peptides in multiple tissues and cell types.

CELF PROTEINS: SPLICING, TRANSLATION AND DM PATHOGENESIS

CELF1 upregulation correlates with muscle histopathology in DM1 patients and DM5 transgenic mice (**Table 1**) (73), pointing to a direct role of CELF1 in disease pathogenesis (**Figure 1**). The upregulation of CELF1 in DM2 skeletal muscle is contentious, with conflicting reports of normal and increased protein levels (33, 74–76).

To directly address the role of CELF1 gain of function, overexpressing mice were generated. Ubiquitous CELF1 upregulation resulted in severe developmental phenotypes and muscle histopathology (77), which correlated with transgene expression levels (77, 78). The high mortality of these mice limited their face and predictive value. Conditional mouse lines were more informative, since they offered the opportunity to focus on individual tissues and assess the pathogenic contribution of CELF protein overexpression alone (**Table 1**). Induction of CELF1 transgene expression in mouse skeletal muscle was sufficient to reproduce muscle wasting, defective motor performance and myopathy (79); while CELF1 upregulation in heart caused cardiac conduction defects, cardiomyopathy with hypertrophy and early mortality (24). As expected, muscular and cardiac phenotypes were accompanied by missplicing events in muscle and heart, respectively (24, 79). A splicing-mediated effect was further supported by the expression of a dominant-negative *Celf1* variant in HSA^{LR} mice, which partially corrected missplicing in skeletal muscle (80).

CELF proteins can also regulate the alternative splicing of transcripts involved in neuronal function (81). Therefore, the upregulation of CELF1 and CELF2 reported in human DM1 brains (27, 82) may have a substantial contribution to the etiology of neurological dysfunction. Conditional overexpressing models could help investigate the cognitive and behavior consequences of CELF1 or CELF2 upregulation, and identify subsets of transcripts that specifically respond to these two RNA-binding proteins. In support of target discrimination between CELF proteins, it was shown that the splicing of *MAPT* exon 10 responds specifically to CELF2, but not to CELF1 upregulation (82).

Although already generated, CELF2-overexpressing mice have only been used in molecular approaches to study the antagonistic role of MBNL and CELF proteins in splicing regulation (25). The phenotypic consequences of CELF2 overexpression have not been reported yet.

Cytoplasmic Functions of CELF Proteins

In addition to regulating alternative splicing in the nucleus, CELF proteins have cytoplasmic roles in the regulation of mRNA stability, translation and deadenylation (83). The distribution of CELF1 between the nucleus and the cytoplasm is regulated by AKT phosphorylation (84). As a result, CELF gain of function in DM1 has intricate consequences that affect multiple cellular pathways in different cell compartments.

CELF1 activity is controlled by multiple phosphorylation events. The role of CELF1 in translation depends on the phosphorylation of Serine-302: phosphorylated CELF1 acts as an activator, while unphosphorylated CELF1 represses translation (84, 85). In DM1, the increase in the total levels of CELF1 is accompanied by the elevation of both phosphorylated and unphosphorylated forms of CELF1 at Serine-302 (40, 86, 87). This results in the reprogramming of protein translation, altered proteostasis and global cell stress, which ultimately affects cell function (75, 78, 84).

DM5 mice have corroborated the cytoplasmic functions of CELF1 in DM1 pathogenesis. The genetic inactivation of *Celf1* in DM5 mice did not mitigate missplicing, but instead corrected the expression of CELF1 translational targets in skeletal muscle (73); a tissue that shows CELF1 upregulation

in DM5 mice (17). The molecular changes were sufficient to improve motor performance, grip strength and histopathology, but left myotonia unchanged (73). These results demonstrate the pathogenic relevance of CELF1-regulated translation, and point to CELF1-independent myotonia mechanisms in DM. Interestingly, in line with the absence of CELF1 upregulation in DM5 hearts (17), *Celf1* deletion did not ameliorate the cardiac function (73).

Finally, it is worth noting that CELF1 overexpression alone is associated with the hyperphosphorylation of Synapsin-1 in cell culture (27) (**Figure 2**), suggesting a contributing role of this RNA-binding protein in the regulation of the phosphoproteome.

Mechanisms of CELF Upregulation and Therapeutic Strategies

CELF1 upregulation in DM1 operates at protein level, since transcript load remains unchanged in skeletal muscle (73). In the heart of DM1 patients and induced EpA960 mice, upregulation correlates with CELF1 protein hyperphosphorylation, higher protein stability and increased PKC activity (87) (**Figure 1**). Consistent with a direct role of PKC in CELF1 metabolism, treatment of EpA960 mice with PKC inhibitors immediately after transgene induction avoided CELF1 upregulation, reduced mouse mortality and improved cardiac function (88). These findings provided pharmacological evidence of the involvement of PKC in CELF1 function and in DM1 cardiac phenotypes. Surprisingly, the genetic inactivation of *Pkc* did not lower CELF1 expression or correct histopathology in the skeletal muscle of DM5 mice (89).

Different reasons may account for the differing outcomes of CELF1 results obtained with EpA960 and DM5 mice. First, the inherent differences between the two mouse models: while in EpA960 interrupted large repeats are expressed under the control of a non-*DMPK* promoter (16), DM5 mice express short (CUG)5 RNA repeat in multiple tissues and cell types under the control of the human *DMPK* promoter (17). Second, it is conceivable that the molecular mechanisms of CELF1 upregulation differ between heart and skeletal muscle: CELF1 upregulation in the skeletal muscle might be independent of PKC. Third, off-target effects of kinase inhibition might have introduced confounding factors in the analysis. Indeed, the PKC inhibitor Ro 31-8220 used in EpA960 mice has since then been found to reduce RNA foci, release MBNL1 and correct MBNL1-dependent splicing events in a cell model of DM1 (90). Furthermore, Ro 31-8220 can also inhibit other kinases, including GSK3ß (91).

Experimental evidence of the role of GSK3ß in DM1 muscle pathology was obtained in HSA^{LR} mice: GSK3ß inhibition restored CELF1 protein levels and translational activity, improved muscle strength and corrected histopathological changes (13, 92). The possibility remains that different aspects of CELF1 metabolism are controlled by different phosphorylation events: phosphorylation by PKC increases protein stability (87, 92), GSK3ß controls the translational activity of CELF1 (13) and AKT regulates the nucleus-cytoplasm distribution of CELF1 (84) (**Figure 1**). We currently do not know the molecular link between the repeat expansion and altered kinase activity.

Kinase-independent mechanisms of CELF1 upregulation have been proposed. Under physiological conditions, CELF1 protein decreases in adult mouse tissues in response to a developmental increase in a subset of microRNA (miRNA) species. Transgene induction in EpA960 revealed that CUG RNA toxicity disrupts the MEF2 transcription network, lowers miRNA expression reversing the physiological miRNA developmental program and causing CELF1 upregulation (93). Therefore altered levels of miRNA in DM1 tissues could explain CELF1 upregulation.

To discard the possibility of a direct or indirect regulation of CELF1 by MBNL proteins, CELF1 expression was measured in *Mbnl1* KO mice, and revealed no changes (18). In contrast, induction of CELF1 over-expression in transgenic mice yielded MBNL1 upregulation, possibly mediated by tissue regeneration (79).

Despite progress in the understanding of the multifaceted metabolism of CELF1 in DM1, the jury is still out on the molecular mechanisms of upregulation in DM1 and the extent of the therapeutic benefits of CELF targeting in tissues, other than the heart. The mechanisms behind CELF2 upregulation in the CNS of DMSXL mice are less clear (27). Useful mouse models are available to address these questions (**Table 1** and **Table 2**), through pharmacological or genetic manipulation of CELF1 and CELF2 levels, as well as the activity of candidate kinases and miRNA metabolism.

UNRAVELING DISEASE INTERMEDIATES AND PATHWAYS BEHIND NEUROMUSCULAR PATHOLOGY

Additional layers of DM1 molecular pathogenesis, beyond the canonical involvement MBNL and CELF RNA-binding proteins, have emerged from recent mouse studies of muscle and heart phenotypes. Hereditary myotonia is usually caused by the malfunction of ion channels (94). In line with this view, compelling evidence has demonstrated the direct role of *CLCN1* chloride channel missplicing in the onset of DM1 and DM2 myotonia (39, 63). The mechanisms behind muscle weakness/wasting and cardiac dysfunction can be more diverse, and mediated by a combination of interacting intermediates. In this section we first discuss some critical splicing events, whose contribution to muscle and heart pathology has been corroborated by mouse studies. Then we review the emerging role of additional pathways, whose mechanistic link with MBNL and CELF canonical disease intermediates has not yet been elucidated and deserves further attention.

The Role of Missplicing in Muscle and Heart Disease: Many Roads Leading to Rome

Progressive muscle weakness and wasting are among the most prominent clinical features of DM1, in association with centralized nuclei and myofiber atrophy, without overt regeneration, fibrosis or necrosis (1). Previous studies have shown associations between muscle weakness and MBNL1-dependent splicing of *BIN1* (95), *CACNA1S* (96) and *DMD*

(97). The recreation of the DM1 missplicing of *Bin1*, *Cacna1s* or *Dmd* in wild-type mice, through RNA antisense technology, corroborated the contribution of these events to muscle weakness and myopathy (95–98). However, it is still unclear if the combined inactivation of multiple MBNL proteins is the sole responsible for muscle weakness. Elevation of CELF1 protein may certainly play a determinant role too, as suggested by the muscle phenotype of CELF1-overexpressing mice (30) and by the improved muscle strength following CELF1 downregulation in HSA^{LR} mice (13). Some CELF1-responsive splicing events may provide connecting dots in the mechanisms of muscle pathology: while *RYR1* missplicing alters excitation-contraction coupling in skeletal muscle (99), the shift of *PKM* splicing to an embryonic isoform results in less efficient energy production, likely associated with muscle weakness and wasting (98).

An expected role for splicing dysregulation has also been suggested in DM heart disease. In spite of the confirmed contribution of MBNL1/MBNL2 loss of function (21) and CELF1 upregulation toward cardiac conduction defects (24), the downstream disease intermediates remained elusive. MBNL1-dependent missplicing of *SCN5A* was found in the heart of DM1 patients and *Mbnl1/Mbnl2* DKO mice. When the DM1 splicing isoform is expressed in wild-type mice, it causes DM1-like cardiac conduction defects and arrhythmias (100). The influential role of SCN5A does not rule out the contribution of other yet unidentified splicing events that may reinforce heart spliceopathy and aggravate cardiac disease in DM.

Cellular Energy Sensors, Proteasome Activity and Muscle Weakness

The RNA binding protein Staufen1 is significantly upregulated in DM1 muscle biopsies, in the absence of missplicing of the corresponding transcript, and it correlates with disease severity (101). Sustained expression of Staufen1 in the skeletal muscle of overexpressing transgenic mice causes muscle weakness and myopathy, characterized by an increase in the frequency of small fibers and central nuclei. Staufen1 impairs muscle differentiation through enhanced translation of c-myc (102), which in turn upregulates the transcription of the *PTEN* tumor suppressor gene and ultimately inhibits downstream AKT signaling (103). The AKT pathway promotes cell survival, proliferation and growth and mediates cell metabolism, transcription and translation in response to extracellular stimuli and changes in energy balance (104). The increased expression of atrogenes in Staufen1-overexpressing mice was linked to AKT signaling inactivation and PTEN upregulation, which interfere with the activity of the ubiquitin-proteasome system to promote catabolic protein degradation, which likely contributes to the muscular phenotypes (103). In further support of elevated protein degradation in DM1 muscle weakness and myopathy, DMSXL mice show enhanced proteasome activity in association with muscle weakness and myopathy (26, 105).

The dysregulation of the adaptive switch between catabolic and anabolic states in DM may extend beyond AKT missignaling, and encompass other intermediates. Maintaining an adequate supply of energy is an essential requirement for cell function, notably in muscle and CNS, which depends on the cross talk between AKT and AMPK signaling pathways (104). Interestingly, the activation of AMPK signaling is also impaired in the skeletal muscle of HSA^{LR} mice following fasting (106), corroborating the idea that DM perturbs cell master sensors of energy balance. Importantly, pharmacological treatments to normalize this pathway improved muscle strength and corrected myotonia in these mice (106). Although these data suggest a role of the AMPK cascade in DM1 muscle pathology, it was also noted that the pharmacological activation of AMPK reduced RNA foci in HSA^{LR} mice. Hence, it is possible that rather than a direct role on the etiology of muscle pathology, AMPK dysregulation perturbs the dynamics of CUG RNA, stabilizes foci and accentuates spliceopathy, thereby aggravating muscle manifestations. Conversely, the AMPK activator alone may simply destabilize RNA foci and lead to an amelioration of mouse phenotypes through a restoration of splicing.

Given the role of Staufen1 in neuronal dendrite arborization and synaptic development (107), it will be of interest to study the implication of Staufen1 in the neurological deficits of DM1. Both AKT and AMPK signaling pathways are implicated in multiple aspects of brain development and function, and their dysregulation has been associated with neurological disease (104, 108). Their role in DM may, however, be restricted to muscle, since no altered AKT/AMPK signaling activity was detected in DM1 neural stem cells (109). Nonetheless, these results must be confirmed in relevant DM mouse models of brain dysfunction.

A Role for Inflammation in Muscle Pathology

Tumor necrosis factor superfamily member 12 (TNFSF12) was found upregulated in the skeletal muscle of DM5 and DM200 mice (**Table 1**), shortly after transgene induction and prior to the onset of muscle pathology (110). Genetic deletion of *Tnfsf12* or the inhibition of the downstream signaling cascade by anti-TWEAK antibodies improved the muscle strength of DM5 mice, demonstrating the physiological relevance of TWEAK signaling in DM1. The binding of TWEAK to its receptor, TNFSF12, regulates cell proliferation, differentiation, inflammation and apoptosis (111). In muscle, the TWEAK-TNFSF12 complex becomes particularly engaged in response to disease, triggering the activation of pro-inflammatory responses that can contribute to DM1 myopathy (110). Further support of ongoing inflammation in muscle was provided by global analysis of gene expression in congenital DM1, which revealed significant upregulation of pro-inflammatory genes (112).

It is conceivable that muscle weakness and atrophy in DM1 is multifactorial process, resulting not only from simultaneous dysregulation of splicing, unbalanced protein synthesis/degradation, but also inflammation.

Changes in miRNA Levels: Defective Transcription or Maturation?

miRNA profiling revealed significant changes in the heart (113), skeletal muscle (114–118) and serum (119) of DM1 and/or DM2 patients. Despite the divergence of some of the results reported,

miRNA dysregulation emerged as a disease feature, which could either be a direct consequence of RNA toxicity, or a lateral event secondary to altered cell physiology. The investigation of miR-1 dysregulation favored the former. Mature miR-1 appears to be downregulated in DM1 and DM2 hearts, in association with an expected increase in miR-1 downstream targets: the upregulation of GJA1 (connexin 43) gap junction protein and CACNA1C calcium channel might subsequently contribute to heart phenotypes (113). In an effort to shed light onto the mechanisms of miR-1 misregulation, MBNL1 knocking down in cell culture blocked the maturation of pre-miR-1, which suggested a role of MBNL1 in miRNA processing and biogenesis, in agreement with the normal or elevated levels of pre-miR-1 found in DM1 and DM2 patients, respectively (113). However, this hypothesis is at odds with subsequent findings. First, miR-1 remained unaltered in *Mbnl1* KO mice (93). It is possible that MBNL2 upregulation in these mice (21), which compensates for the lack of MBNL1, could avoid miR-1 downregulation. To answer this question it would be important to study miR-1 levels in *Mbnl1/Mbnl2* DKO. Second, global analysis of miRNA species revealed that CUG-associated changes occurred already at the precursor stage in the induced EpA960 mouse model, arguing against a primary defect in subsequent miRNA processing and maturation. Instead, these results were consistent with defects in miRNA transcription and were attributed to the dysregulation of the MEF2 transcriptional program (93). It is possible that the high expression levels of the expanded (and interrupted) transgene in EpA960 mice trigger severe molecular defects and more pronounced dysregulation of miR-1 transcription, upstream from processing and maturation, relative to DM1 and DM2 patients. Finally, recent findings on CELF1-overexpressing mice did not fully match previous results in human tissue either. In contrast with the upregulation of miR-1 targets reported in DM1 and DM2 hearts (113), GJA1 protein levels decrease in the heart of CELF1-overexpressing mice (120). The discrepancy between patients and these mice might be explained by a combined effect of the heterogeneous regional distribution of GJA1 in disease hearts, and the study of different disease stages: GJA1 levels may show an initial compensatory increase during the early adaptation disease stages studied in human samples (113), followed by a late decrease during maladaptation disease stages, like in CELF1-overexpressing mice (120). Further studies are required to clarify these questions and to extend the implications of miRNA metabolism to other affected tissues, notably the CNS.

DM1 Cardiac Function: Revisiting DMPK Loss of Function

The sequestration of expanded *DMPK* RNA in the nucleus of DM1 cells causes a 50% reduction in protein levels (121). Initial reports suggested a role of *DMPK* haploinsufficiency in disease etiology, a hypothesis corroborated by a dose-dependent effect in mouse heart: the deletion of one copy of the murine *Dmpk* gene was sufficient to disrupt cardiac conduction (122, 123). In contrast, late and mild myopathy in skeletal muscle required full deletion of both *Dmpk* copies in knockout mice (124). These early findings suggest that therapeutic hopes aiming to eliminate *DMPK* transcripts may aggravate some aspects of the disease pathology, particularly in heart. In this context, it is worth reviewing our actual knowledge on the contribution of DMPK protein to disease.

The recent re-evaluation of the impact of *Dmpk* deletion in knockout mice, bred onto homogeneous genetic backgrounds, showed no functional impact on cardiac or skeletal muscle, thereby excluding a role of DMPK loss of function in muscle phenotypes (125). The reasons behind the diverging results relative to early findings may relate to the strain background and the role of unidentified modifiers. Alternatively, the differences may relate to the replacement strategy used to inactivate the *Dmpk* gene, which might have interfered with the expression of flanking genes in the knockout lines previously generated (125). In summary, these data provide evidence of the limited functional impact of *DMPK* inactivation on heart and skeletal muscle, and validate the anti-sense therapies being developed, which are discussed below. Nonetheless, the role of DMPK protein in the CNS, as well as in other tissues, needs to be further explored.

DM MOUSE MODELS OF NERVOUS SYSTEM DYSFUNCTION

The burden of CNS dysfunction has shifted DM research from an initial focus on muscle pathology, to the investigation of brain disease mechanisms. Sophisticated imaging techniques have characterized structural and metabolic abnormalities in human brains (2, 126). Molecular studies have also been performed in the nervous system, but they rely on samples collected at the end-stage of the disease. Animal models overcome this critical limitation, as they provide tissue samples throughout disease progression, offering the possibility to characterize molecular, cellular and electrophysiological changes in the nervous system prior to the onset of disease symptoms. In this section we critically review relevant neurological phenotypes of various DM mouse models, and the insight they provide to the understanding of disease mechanisms in the central and peripheral nervous system.

The Expression of Toxic RNA in the CNS

Two DM1 mouse models express large CUG RNA transcripts in the CNS: the ubiquitous DMSXL line and the inducible EpA960 mice (**Table 1**). Both DMSXL and forebrain-induced EpA960 mice show impaired spatial learning and memory in the Morris Water Maze, resembling the visuoconstructive defects in DM1 patients (27, 31). DMSXL mice have also shown signs of anhedonia and novelty inhibition of exploratory activity (27). The electrophysiological profiling of the hippocampus revealed synaptic dysfunction behind these phenotypes: while DMSXL mice show impaired short-term paired-pulse facilitation (27), suggestive of pre-synaptic dysfunction; EpA960 exhibit reduced long-term potentiation (LTP) (31), which is more often associated with post-synaptic abnormalities (**Figure 2**). The diverging effects on pre- and post-synaptic neuronal plasticity between the mouse lines may be accounted for, at least partly, by their intrinsic differences: DMSXL mice express pure CUG repeats in multiple brain cell types from an early embryonic

stage; while induced EpA960 mice express higher levels of interrupted CUG repeats post-natally, in the neurons of the forebrain (**Table 1**).

Typical RNA foci accumulation and co-localization with MBNL1 and MBNL2 were detected in various cell types of DMSXL brains (27) and in EpA960 neurons (31). Still, both lines showed only limited spliceopathy (27, 31). In contrast, *Mbnl2* KO and *Mbnl* DKO displayed more pronounced splicing dysregulation, which may contribute to impaired LTP and spatial learning of *Mbnl2* knockout mice (19, 59): the missplicing of *Grin1* may reduce dendritic localization of the glutamate receptor, which may be further aggravated by *Tanc2* abnormalities (127, 128); while *Cacna1d* and *Ndrg4* misregulation might impair neuronal activity and learning (129, 130) (**Figure 2**). Other MBNL-dependent pathways may, however, contribute to brain disease, such as defects in APA (59) and changes in the expression and phosphorylation of synaptic proteins (27, 50, 54).

MAPT/Tau protein has long been associated with DM1 brain disease. Abnormal MAPT isoform distribution was first described at the protein level (131), in association with the intranuclear accumulation of hyperphosphorylated protein fibers, or tangles in patients (**Figure 2**). Abnormal missplicing was later described in patients (46, 132) and in the brain of DMSXL mice (27). The pronounced *Mapt* RNA missplicing in *Mbnl1/Mbnl2* DKO indicates the critical role of the spliceopathy resulting from the dual loss of these two RNA-binding proteins (59). The DM1 tauopathy has been suggested to interfere with axonal transport and neurosecretion (133), but further animal studies are required to decipher the mechanisms.

REGIONAL DISTRIBUTION OF DM PATHOLOGY IN THE BRAIN

Imaging and neuropsychological assessment have uncovered candidate brain regions primarily affected by DM. The identification of critical brain areas will be important to direct future therapies toward the most relevant brain territories, and it will likely depend on an intricate interplay of factors, such as somatic repeat length, levels of toxic RNA, foci abundance and the activity of RNA-binding proteins. A small number of studies has investigated repeat instability (132) and *DMPK* gene expression in different brain areas in a limited number of human patients (134). DM1 mice offer the possibility to surmount the limited availability of human tissue and perform more detailed analyses. Transgenic DM1 mice expressing ∼500 CTG repeats under the control of the human *DMPK* promoter and the regulatory regions of the DM1 locus (14) showed age-dependent accumulation of larger repeat sizes in most brain regions (**Figure 3**). The semi-quantitative results did not reveal brain regions with exceptionally high somatic mosaicism, in which we could anticipate the accumulation of very long CUG repeats. The cerebellum, however, exhibited lower levels of somatic instability, as reported in humans (135) and in another model of CTG repeat instability (136). The average repeat size in the cerebellum was nonetheless within the disease-associated

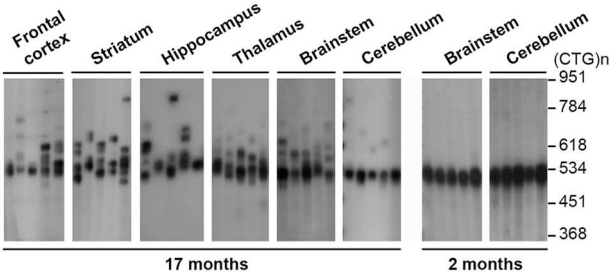

FIGURE 3 | Analysis of CTG somatic mosaicism in the CNS of transgenic mice carrying the DM1 locus. The autoradiographs show representative SP-PCR analyses of 10–20 transgene molecules per reaction in dissected brain regions of old and young DMSXL hemizygotes, aged 17 and 2 months, respectively. The size markers, converted into repeat number are displayed on the right.

range. It is possible that future analyses of somatic repeat instability in smaller brain areas or individual cell types of these mice will reveal susceptible cell populations that accumulate significantly longer repeat expansions.

Similarly, the expression levels of the *DMPK* transgene showed modest variation between CNS regions (54). In contrast, RNA foci were not homogenously distributed and accumulated preferentially in the frontal cortex and certain areas of the brainstem of DMSXL mice (26, 54), and they appeared to be more abundant in cortical astrocytes relative to neurons (27). The analysis of well-defined histological layers of the mouse cerebellum has also shown greater foci accumulation and more severe spliceopathy in Bergmann astrocytes, relative to the neighboring Purkinje cells (50). Together these findings demonstrate more pronounced pathologic events in defined brain cell populations and cell types, a view further supported by the preferential accumulation of anti-sense RAN-translated products in the oligodendrocytes of DM2 brains (70).

The factors governing the distribution of DM pathology in the brain remain elusive and must be addressed in future mouse studies, but variations in the expression of MBNL and other RNA-binding proteins between brain regions (54) and cell types should be considered (50).

THE ROLE OF GABA, GLUTAMATE AND GLIA IN DM1 NEURONAL HYPEREXCITABILITY

While waiting for efficient gene therapy to correct the causing genetic defect (the DNA repeat expansion) or neutralize the pathogenic molecule (the toxic RNA), one can imagine pharmacological means to ameliorate or prevent progression of neurological symptoms. Such strategies require comprehensive characterization of neuronal activity and integrative brain dysfunction.

Perturbed balance between excitatory and inhibitory neurons disrupts cognition in neurological diseases. Several mouse studies favor a scenario of neuronal excitability in DM1. Both DMSXL and *Mbnl2* KO mice present elevated

susceptibility to PTZ-induced seizures, suggesting GABA-mediated hyperexcitability (19). In line with elevated neuronal excitability, *Mbnl2* KO mice show augmented responsiveness to intracortical train stimulation, in a mechanism partially mediated by abnormal glutamate neurotransmission (137). Reduced expression of the glial GLT1 glutamate transporter in DMSXL brains is associated with elevated neuronal firing *in vivo* (50) (**Figure 2**). This finding supports a role of defective glutamatergic transmission and neuronal excitability in DM1, mediated by abnormal neuroglial interactions. Neuronal hyperexcitability is a frequent cause of epilepsy. Although epileptic episodes are rare in DM, patients present high sensitivity to GABA agonists (138) as well as abnormalities in glutamatergic transmission in the frontal lobe (139).

In addition to GABA and glutamate, circumstantial evidence points to the involvement of other signaling molecules. HPLC quantification revealed region-specific defects in dopamine and serotonin neurochemicals in DMSXL brains, in association with high foci content in dopaminergic and serotonergic brain centers (27). Importantly, DM1 brains have shown loss of neurons signaling through these two types of neurotransmitters (140, 141).

Today optogenetics allows the neuronal manipulation of neuronal circuits *in vivo*. In combination with electrophysiology, imaging and behavior assays, these techniques can provide insight into the contribution of neuronal activity to the cognitive performance of DM mice, and elucidate the neuronal circuits most profoundly affected by the disease.

Structural, Developmental and Functional Features of DM Brains: Insight From Mouse Models

The brain structural changes found in DM1 and DM2 are mainly characterized by white matter hyperintensities, some general atrophy and dispersed gray matter reduction across the four cortical lobes, the basal ganglia, and cerebellum. Importantly, white matter abnormalities correlate with disease duration and cognitive deficits (2, 126). Functional imaging revealed low glucose uptake and cerebral hypoperfusion, as well as abnormal connectivity patterns that correlate with atypical personality traits and executive dysfunction (142, 143). The correlation between imaging data and neuropsychological profiles hints to the involvement of complex neuronal networks, through defective neurodevelopment, neurodegeneration or neurodysfuntion. Today we still do not know the contributing weight of each of these components to DM brain disease. The molecular and histological mouse studies have shed some light on this question.

Higher expression of embryonic splicing isoforms in the brains of DMSXL (27), *Mbnl2* KO and *Mbnl1/Mbnl2* DKO mice (19, 59) points to a disrupted developmental program. In contrast, the dysregulation of synaptic proteins does not recreate embryonic events (54), supporting functional deficits in DM brains, rather than a developmental delay.

Inducible EpA960 mice have recently given further insight. Transgene induction in adult forebrain (after the completion of CNS development) yielded progressive loss of axonal and dendritic integrity, together with brain atrophy (31)—a sign of ongoing neurodegeneration in adults, possibly in line with the reported premature and accelerated cognitive decline in DM1 patients (144). However, the EpA960 mouse data do not exclude developmental disruption, should toxic RNA be expressed during early embryonic stages.

Understanding the contribution of defective development, neurodysfunction and neurodegeneration is critical to design therapeutic schemes: we must intervene prior to the establishment of irreversible developmental defects, irreparable cell damage or permanent network dysfunction. Mouse models, and in particular the inducible lines (**Table 1**), will help assess the reversibility of neurological phenotypes and whether neurological disease progression can be halted and even reversed.

The Peripheral Nervous System and the Neuromuscular Junction

The involvement of the peripheral nervous system (PNS) and the presence of peripheral neuropathy in DM1 has been open to debate (145). The scarce availability of human samples has slowed down research on this topic, but mice expressing toxic CUG repeats in the PNS and in the neuromuscular junction (NMJ) have surpassed this limitation.

Axonopathy was detected in the DMSXL sciatic nerves, characterized by smaller nerve sections, loss and reduced size of myelinated fibers, in association with thinner myelin sheaths, which may highlight ongoing pathogenicity in myelinating cells. The neuronopathy extends to the spinal cord of DMSXL, where a reduction in the number of motor neurons was reported (146).

The analysis of the neuromuscular junction (NMJ) in DM1 muscle biopsies revealed abundant accumulation of RNA foci both in pre-synaptic motoneurons and in post-synaptic nuclei, with pronounced MBNL1 sequestration (147). As a result the NMJ is at risk of developing DM1-associated spliceopathy, but we currently do not know which MBNL1-dependent targets and pathways are dysregulated. In addition, the expression of two members of the SLITRK family of membrane proteins is dysregulated in DM1, in a MBNL1-independent manner, affecting neuromuscular connections (148). Together these findings suggest that both MBNL-dependent and MBNL-independent mechanisms may disturb the organization, stability and function of the NMJ, thereby contributing to PNS pathology and, importantly, to muscle pathology. In support of this view, the expression of expanded CUG RNA in the diaphragmatic NMJ of DMSXL mice is associated with disorganized endplates, lower density of postsynaptic acetylcholine receptors and reduced number of myelinated neurons, possibly mediating the respiratory impairment of these mice (28). In contrast, HSA^{LR} transgenic mice exhibit poor foci accumulation in subsynaptic nuclei (147), indicating that the muscle phenotypes of this line (such as myotonia, central nuclei and ring fibers) do not require the expression of toxic RNA in the NMJ. Subsynaptic RNA toxicity in the NMJ would preferably contribute to DM1 muscle features that are not detected in HSA^{LR} mice, such as angular fiber atrophy and pyknotic nuclear clumps (147). In conclusion,

defective communication between nerve endings and skeletal muscle might be a common feature in DM1, likely contributing to muscle pathology.

THERAPY DEVELOPMENT: PRE-CLINICAL MOUSE STUDIES

Following the identification of CUG repeats as the pathogenic element in DM1, expanded RNA transcripts became an attractive therapeutic target, endorsed by the reversion of disease phenotypes in an inducible mouse model of DM1 (17). Hence, the neutralization of CUG repeats has been tested in relevant DM1 mouse models, taking advantage of antisense oligonucleotides (ASO) or small molecules (**Table 3**).

Antisense Oligonucleotides to Neutralize Toxic RNA

ASO have been designed to disperse nuclear RNA foci and redistribute MBNL proteins, or to induce the degradation of expanded transcripts. Early approaches aimed to destabilize CUG RNA foci by direct injection of morpholino-type ASO into the skeletal muscle of HSA^{LR} mice. The reduction in nuclear foci, redistribution of MBNL1 protein and splicing correction was sufficient to improve muscle histology and myotonia (150). Similarly, 2′-O-methyl phosphorothioate (PS) modified ASO reduced foci number and corrected missplicing in two independent mouse models (149); unfortunately the molecular benefits were insufficient to improve muscle phenotypes (**Table 3**). Both strategies reduced the levels of toxic transcripts without RNase H activation, likely through the degradation of expanded transcripts released from nuclear foci. Alternative approaches used RNase H-active ASO to enhance nuclear RNA degradation of CUG repeats. Intramuscular injection and electroporation of 2′-O-methoxyethyl (MOE) gapmers knocked-down expanded CUG transcripts in EpA960 mice and reduced RNA foci (151). Further reduction in toxic RNA was achieved by the combination of RNase H-active MOE gampers and morpholinos (151). However, local injection caused some degree of muscle damage, which aggravated histopathology and splicing dysregulation in these mice. The systemic delivery of ASO overcomes this problem and is particularity attractive given the vast number of tissues and organs affected in DM1: systemic administration of MOE gapmers reduced expanded CUG RNA, corrected global transcriptome, ameliorated histopathology and resulted in long-term suppression of myotonia in HSA^{LR} mice (12). Similarly, 2′-4′-constrained-ethyl (cEt) ASO administrated systemically yielded robust reduction of expanded *DMPK* transcripts, improved body weight, muscle strength and histology of DMSXL mice (154). The demonstration that expanded CUG RNA is a potential target for the RNA interference (RNAi) pathway (163) suggested the therapeutic use of siRNA. Both intramuscular injection and viral delivery of siRNA molecules activated toxic CUG degradation, reduced molecular signs of RNA toxicity and improved the phenotypes of HSA^{LR} mice (152, 155).

ASO offer today a promising pipeline for therapeutic development, but their efficient delivery and biodistribution are still critical hurdles to overcome.

Ligands and Small Molecules to Disperse RNA Foci

Small soluble chemicals with high biodistribution and low toxicity may provide an alternative to ASO. Some of these compounds were tested in DM1 mouse models (**Table 3**). Derivatives of pentamidine (and other diamidines), hoescht and aminoglycoside, as well as synthetic peptides yielded limited correction of missplicing in HSA^{LR} mice (156, 159, 160). While diamidines inhibit the transcription of toxic CUG RNA, the others likely disrupt RNA-protein complexes, releasing MBNL proteins from nuclear CUG foci. Although the benefits of some of these molecules were modest in mice, the results established a scaffold for chemical redesign to optimize biodistribution, reduce toxicity and increase efficacy.

Approaches limited to restoring MBNL function are unlikely to fully address the consequences of RNA toxicity and additional intermediates should also be targeted. The dissection of the molecular pathways implicated in DM1 pathogenesis revealed some of these targets and hinted at novel routes of pharmacological intervention (**Table 3**). In the future, therapeutic combination of multiple approaches to eliminate the primary offending RNA with approaches to correct downstream pathogenic events might be required.

DNA as Therapeutic Target

Strategies targeting the DNA repeat expansion mutation were previously tested in DM1 mouse cell culture systems (164) or directly in HSA^{LR} skeletal muscle (165), and proved capable of stabilizing the trinucleotide CTG repeat tract. Although substantial effort has concentrated on the deleterious accumulation of toxic RNA, recent gene editing tools provide new means to target the upstream DNA mutation that causes DM1. CRISPR/Cas9 systems were tested in DMSXL mouse cells to induce repeat contractions (43), while modified Cas9 was used in HSA^{LR} mice to block the transcription of toxic RNA (166).

DM1 ANIMAL MODELS BEHIND MICE

By definition, an animal model provides a simplification of the complex human system, or at least, part of it. Mouse models offer a good compromise between easy manipulation, affordable research cost and similarity to the complex physiology of humans. However, mice have limitations too, and today there is no perfect DM1 mouse model that fully recreates all disease aspects. Conversely, reduced body mass has been repeatedly reported in mice (21, 23, 26, 77, 78) but no direct parallel has been established with human clinical symptoms, nor is it known to what extend this phenotype reflects a DM1-associated developmental delay.

Given the nature of the constructs used, transgene expression varies between models and introduces some drawbacks that should not be overlooked. Some constitutive models (such as the DMSXL mice) express low transgene levels, and require

TABLE 3 | Therapeutic strategies tested in DM1 mouse models.

Compound	Target	Administration	Mechanism	Mouse model	Benefits reported in DM1 mice	References
ANTISENSE OLIGONUCLEOTIDES						
PS	CUG sequence	Intramuscular injection (local)	Steric hindrance and foci dispersion	DMSXL[a] HSA^{LR}	Dispersion of RNA foci Reduction of CUG RNA Splicing correction	(149)
Morpholino	CUG sequence	Intramuscular injection and electroporation (local)	Steric hindrance and foci dispersion	HSA^{LR} DMSXL[a]	Reduction of CUG RNA Splicing correction Mitigation of myotonia	(150)
MOE-gapmer	Flanking region	Subcutaneous injection (systemic)	RNase H-mediated degradation	HSA^{LR}	Reduction of CUG RNA Splicing correction Improved histology Sustained mitigation of myotonia	(12)
MOE-gapmer	CUG sequence	Intramuscular injection and electroporation (local)	RNase H-mediated degradation	Induced EpA960	Dispersion of RNA foci Splicing correction	(151)
MOE-gapmer and morpholino	CUG sequence	Intramuscular injection and electroporation (local)	Combined RNase H and foci release	Induced EpA960	Enhanced reduction of CUG RNA	(151)
siRNA	CUG sequence	Intramuscular injection and electroporation (local)	RNAi-mediated RNA degradation	HSA^{LR}	Reduction of CUG RNA Dispersion of RNA foci Splicing correction Mitigation of myotonia	(152)
cEt gapmer	*DMPK* 3′UTR	Subcutaneous injection (systemic)	RNase H-mediated degradation	DMSXL	Reduction of CUG RNA Body weight gain Improved muscle strength Improved histology	(153, 154)
siRNA	hACTA1 3′UTR	Intravenous injection of rAAV vectors (systemic)	RNAi-mediated RNA degradation	HSA^{LR}	Reduction of CUG RNA Splicing correction Improved histology Mitigation of myotonia	(155)
NUCLEIC ACID BINDING CHEMICALS						
Pentamidine, heptamidine and diamidine analogs	CUG transcription	Intraperitoneal injection (systemic)	Inhibition of CUG transcription Foci dispersion and CUG RNA degradation	HSA^{LR}	Reduction of CUG RNA Splicing correction Mitigation of myotonia	(156–158)
Hoescht derivatives	CUG-MBNL complex	Intraperitoneal injection (systemic)	Disruption of RNA foci	HSA^{LR}	Splicing correction	(159)
Kanamycin derivatives	CUG-MBNL complex	Intraperitoneal injection (systemic)	Disruption of RNA foci	HSA^{LR}	Splicing correction	(160)
Synthetic peptide	CUG-MBNL complex	Intramuscular injection (local)	Disruption of RNA foci	HSA^{LR}	Improved histology Splicing correction	(161)
Actinomycin D	CUG transcription	Intraperitoneal injection (systemic)	Inhibition of CUG transcription	HSA^{LR}	Reduction of CUG RNA Splicing correction	(162)
PHARMACOLOGICAL APPROACHES						
Ceftriaxone	GLT1, glial glutamate transporter	Intraperitoneal injection (systemic)	Upregulation of GLT1	DMSXL	Correction of Purkinje cell firing Improved motor coordination	(50)
Bio, Lithium, TDZD-8	GSK3ß	Intraperitoneal injection (systemic)	GSK3ß inhibition	HSA^{LR}	Improved histology Improved muscle strength Mitigation of myotonia	(13)
Ro-31-8220	PKC	Intraperitoneal injection (systemic)	PKC inhibition	EpA960	CELF1 downregulation Splicing correction Amelioration of cardiac function	(88)
AICAR	AMPA signaling	Intraperitoneal injection (systemic)	AMPK activation	HSA^{LR}	Dispersion of RNA foci Splicing correction Mitigation of myotonia	(106)
Rapamycin and AZD8055	mTOR signaling	Intraperitoneal injection (systemic)	mTORC1 inhibition	HSA^{LR}	Improved muscle function and strength	(106)
Anti-TWEAK antibody	TWEAK/Fn14 signaling	Intraperitoneal injection (systemic)	TWEAK	DM5	Improved muscle histology Improved muscle strength Greater survival	(110)

rAAV, recombinant adeno-associated viral; ASO, antisense oligonucleotide; BIO, 6-bromoindirubin-39-oxime; cEt, 2′,4′-constrained ethyl-modified; LNA, locked nucleic acids; MOE, 2′-O-methoxyethyl; PS, 2′-O-methyl phosphorothioate. [a]The DMSXL mice used in these studies were hemizygous and carried ∼500–800 CTG.

breeding to homozygosity to develop disease phenotypes. In contrast, the high expression levels in tissue-specific models (such as the EpA960 and DM5 mice) may trigger some non-specific disease features. Finally, tissue and cell type-specific expression in HSA^{LR}, EpA960 and DM5/DM200 mice can mask non-cell-autonomous mechanisms, critical for some features of disease pathogenesis. In summary, the collection of mice available today covers different aspects of DM1 pathology to a certain extent, partially fulfilling the absence of a perfect mouse model, and providing means for data validation by independent laboratories.

Simple organisms can also provide complementary models for basic, translational and pre-clinical research. Although phylogenetically distant from humans, *Drosophila melanogaster, Caenorhabditis elegans* or zebrafish (*Danio rerio*), have multiple advantages over mice, including their easy manipulation, low maintenance cost and fast generation of large offspring. The expression of toxic RNA in *D. melanogaster* recreated molecular features of DM1, such as RNA foci accumulation, muscleblind protein sequestration and missplicing (167–169). Some lines showed eye degeneration (167, 168), a general readout of neurotoxicity, but which does not necessarily relate to human pathology. The development of muscle phenotypes, such as muscle wasting (167) and hypercontraction (169) seems more relevant. The expression of expanded CUG repeats also resulted in RNA foci and muscle phenotypes in zebrafish (170, 171) and *C. elegans* (172, 173). Together, these data suggest the conservation of the core mechanisms of RNA toxicity across species, and corroborate the use of simple organisms in large screenings for disease modifiers. Such studies have already resulted in the identification if genetic modifiers (167, 174, 175), chemicals that correct DM1 splicing abnormalities (176) and miRNA sponges that regulate MBNL protein levels and rescue fly phenotypes (177). The physiology of small organisms and humans are nonetheless substantially different, and therefore parallels must be established with care.

CONCLUSIONS

Transgenic mouse models, alone or in combination, have been key to understanding fundamental molecular pathomechanisms of DM. Over the last decade, the progress in mouse studies and the advances in high throughput approaches (e.g., transcriptomics and proteomics) have led to the identification of hundreds of misregulated genes and proteins, through changes in alternative splicing, polyadenylation, protein translation and phosphorylation. Understanding the contribution of these molecular events to the etiology of DM will help depict the course between repeat expansion and the onset of disease manifestations. Future studies should continue to address "which" disease intermediates and cell populations, "where" in the tissue and "when" during disease course experience the most pronounced abnormalities. Linking these variables will identify critical events and developmental windows during which specific cell pathways are particularly sensitive to pathological insults and targetable by corrective therapies. A better understanding of pathophysiological trajectories will guide the development of efficient therapeutic approaches.

Some models have deliberately focused on specific disease features and recapitulated a small number of disease phenotypes (e.g. muscle pathology in HSA^{LR} models, cardiac function in inducible CELF1-overexpressing mice). Although oversimplifying the situation, this reductionist approach has offered the opportunity to break the complexity of disease down to tractable "building blocks" and to unravel the mechanisms behind individual aspects of the disease. The future combination of these different models, by intercrossing different transgenic lines, might be considered to "rebuild" the convoluted human disease and to explore the interdependence of individual factors. The complexity of DM pathobiology and variation in mouse models design require, however, a critical approach in the interpretation and comparison of the results obtained with different lines.

There is little doubt that mouse models will continue to provide in-depth understanding of disease. One of their major advantages is the opportunity to monitor early pathological changes, prior to the onset of disease symptoms, which is difficult to achieve in humans with the current diagnostic standards. We anticipate that future studies will uncover additional cellular pathways impacted during the disease course, while revealing targetable events to reverse disease.

AUTHOR CONTRIBUTIONS

JA: experimental work and data acquisition; GG and MG-P: study design, interpretation, data analysis; SB and MG-P: preparation of figures; SB, GG, and MG-P: manuscript preparation.

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MINI REVIEW
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Modeling of Myotonic Dystrophy Cardiac Phenotypes in *Drosophila*

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After respiratory distress, cardiac dysfunction is the second most common cause of fatality associated with the myotonic dystrophy (DM) disease. Despite the prevalance of heart failure in DM, physiopathological studies on heart symptoms have been relatively scarce because few murine models faithfully reproduce the cardiac disease. Consequently, only a small number of candidate compounds have been evaluated in this specific phenotype. To help cover this gap *Drosophila* combines the amenability of its invertebrate genetics with the possibility of quickly acquiring physiological parameters suitable for meaningful comparisons with vertebrate animal models and humans. Here we review available descriptions of cardiac disease in DM type 1 and type 2, and three recent papers reporting the cardiac toxicity of non-coding CUG (DM1) and CCUG (DM2) repeat RNA in flies. Notably, flies expressing CUG or CCUG RNA in their hearts developed strong arrhythmias and had reduced fractional shortening, which correlates with similar phenotypes in DM patients. Overexpression of Muscleblind, which is abnormally sequestered by CUG and CCUG repeat RNA, managed to strongly suppress arrhythmias and fractional shortening, thus demonstrating that Muscleblind depletion causes cardiac phenotypes in flies. Importantly, small molecules pentamidine and daunorubicin were able to rescue cardiac phenotypes by releasing Muscleblind from sequestration. Taken together, fly heart models have the potential to make important contributions to the understanding of the molecular causes of cardiac dysfunction in DM and in the quick assessment of candidate therapeutics.

Keywords: cardiac dysfunction, myotonic dystrophy, Muscleblind, CTG expansion, CCTG expansion, *Drosophila* disease model, drugs

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INTRODUCTION

Myotonic Dystrophy (DM) is characterized by autosomal dominant inheritance and multisystem involvement. Progressive myotonia, muscle degeneration, early onset cataracts, heart defects, neurological problems and endocrine disorders are the most observed multisystemic dysfunctions (1, 2). To date, two distinct forms of DM have been identified. DM1 is caused by an unstable CTG repeat expansion in the 3′UTR of the DMPK gene (OMIM 605377) (3–5). DM2 is caused by an abnormal CCTG expansion in the first intron of the CNBP gene [previously known as zinc finger 9 gene, ZNF9; OMIM 116955] (6–8). Both types share the common disease characteristics, however, they also have distinct clinical features. Prominent distal muscle involvement, marked myotonia and severe congenital form are seen in DM1

whereas DM2 is characterized by prominent proximal muscle involvement, mild myotonia, and absence of congenital form. Clinically, DM2 is milder than DM1 (9). At the molecular level the mutant transcripts [C(C)UG] accumulate in foci leading to disruption of key cellular pathways, namely, RNA processing (10), localization (11), and translation (12, 13). These mutant transcripts alter the muscleblind-like and CUGBP and ETR3 like factor families of RBPs and results in abnormal expression of fetal isoforms of several genes in adult tissues (14, 15). In addition, deregulation of microRNA and RAN-translation may be important additional mechanisms of DM pathophysiology (16–19). Different vertebrate and invertebrate animal models have been successfully generated by different laboratories to understand the disease pathomechanisms. Most of the animal models have been paramount to understand the muscle-related pathomechanisms (20–22). However, till date, only a few reports are available about animal models to study DM heart problems (23, 24). Interestingly, *Drosophila* has been shown to mimic DM cardiac dysfunctions (25, 26). The purpose of this review is to gather all the available information about *Drosophila* cardiac dysfunction models in DM, which are found to complement functional data coming from murine models.

Heart-Related Alterations in DM1

Approximately 80% of the DM1 patients will develop the cardiac disease in their lives but the risk is more pronounced in young patients (2–30 years old) than in the old ones (27). Indeed, the cardiac complications account for 30% of patient deaths (28–30). The cardiac involvement mainly includes degeneration of conduction system caused by myocardial fibrosis (31). Myocardial fibrosis is due to myocyte hypertrophy, focal fatty infiltration, and also lymphocytic infiltration (32, 33). This are affecting 40% of the DM1 patients (34) and 65% patients have an abnormal ECG. The typical ECG abnormalities include prolongation of PR interval (>240 ms; 20–40% patients) and the QRS duration (>120 ms; 5–25% DM1 patients) (35).

Conduction disturbances can cause conduction block, ectopic activity, and re-entrant arrhythmias. These disturbances give rise to palpitations, syncope and sudden cardiac death (36). Both atrial and ventricular arrhythmias can occur in DM1 patients. Around 25% of the DM1 patients show atrial (supraventricular) tachyarrhythmias, specifically atrial fibrillation and atrial flutter (30, 34). Ventricular arrhythmias are less common but more severe and are considered as the main cause of sudden death (37, 38).

DM1 patients are also prone to develop structural cardiomyopathy (39, 40). Early in the disease course, the left ventricular diastolic dysfunction is more pronounced than the systolic dysfunction (41). In addition, left atrial dilatation may also occur (33). This impaired relaxation of the cardiac muscle or myocardial myotonia is the cardiac equivalent of skeletal muscle problems in patients (30, 41). Other associated heart manifestations include angina (both stable and unstable), and myocardial infarction. Mitral valve prolapse has been identified in 13–40% of patients and was directly related to stress-induced ejection fraction problem. In some DM1 patients, pulmonary failure was also observed (42).

Heart-Related Problems in DM2

Generally, heart dysfunction in DM2 was considered less severe and frequent than in DM1 (43–45). However, recent studies indicate that the total risk of cardiac disease in DM2 is very close to DM1 (9). Like in DM1, DM2 cardiac manifestations include AV blocks, arrhythmias, and dilated cardiomyopathy (46). The subclinical myocardial injury causes conduction defects and is directly correlated with the ECG abnormalities found in DM2 patients (42). Conduction defects also cause severe arrhythmias and sudden death in DM2 patients (40). In contrary to DM1, DM2 patients do not show pulmonary failure (42).

Murine Models to Study Heart Dysfunction in DM

Different mice models have been created to understand the cardiac aspect of the disease. These are, (1) overexpression of expanded (DMSXL) (24) or (2) non-expanded (Tg26) DMPK (47), (3) Cre-lox inducible heart-specific expression of CUG repeats [EpA960] (23), (4) inducible expression of DMPK 3'UTR with short repeats [GFP-DMPK-(CTG)5] (48), (5) compound loss of Mbnl1 and Mbnl2 (49), and (6) CUGBP1 overexpressing mice (50). All of these mice models reproduce DM1-specific cardiac dysfunction to some extent but they do have some specific limitations. EpA960 mice have shown DM1-like ECG recordings, arrhythmia and AV block, but they were so seriously affected that died very early. The DMSXL mice reproduced important clinical aspects as observed in the disease including reduced muscle strength, lower motor performance, and respiratory impairment, but cardiac phenotypes of DMSXL required challenging by the class-I antiarrhythmic agent flecainide (51). In addition, missplicing defects were mild. The GFP-DMPK-(CTG)5 mice showed toxicity within the normal range of repeats in the absence of ribonuclear foci, and had a high rate of mortality. Finally, Mbnl loss of function or CUGBP1 overexpression is not representative of the disease complexity. Therefore, investigation of physiopathological pathways and testing of drugs still needs development of additional whole animal models.

The *Drosophila* Heart as Alternative to Vertebrate Cardiac Models

The *Drosophila* heart has remarkable similarities with vertebrates in terms of structure and developmental regulation. A common developmental origin has been found on bilaterally symmetrical rows of mesodermal cells which migrate and fuse to form a heart tube at the midline (52). Upon subsequent looping and septa formation, the fly heart is further divided by an intracardiac valve into an aorta and a 1 mm long pulsatile posterior dorsal vessel or proper heart (**Figure 1A**) (54–56). The *Drosophila* heart also possesses a bilateral pacemaker system. The chief pacemaker situated near aorta expels hemolymph anteriorly whereas, the minor pacemaker placed in conical chamber allows backtracking of hemolymph flow (53, 57, 58). Nevertheless, the mechanism behind the origin of pacemaker potential has not been elucidated (58). So making direct extrapolation of *Drosophila* results to mammals is difficult. In contrast, the fly heart is different in two critical aspects. Calcium

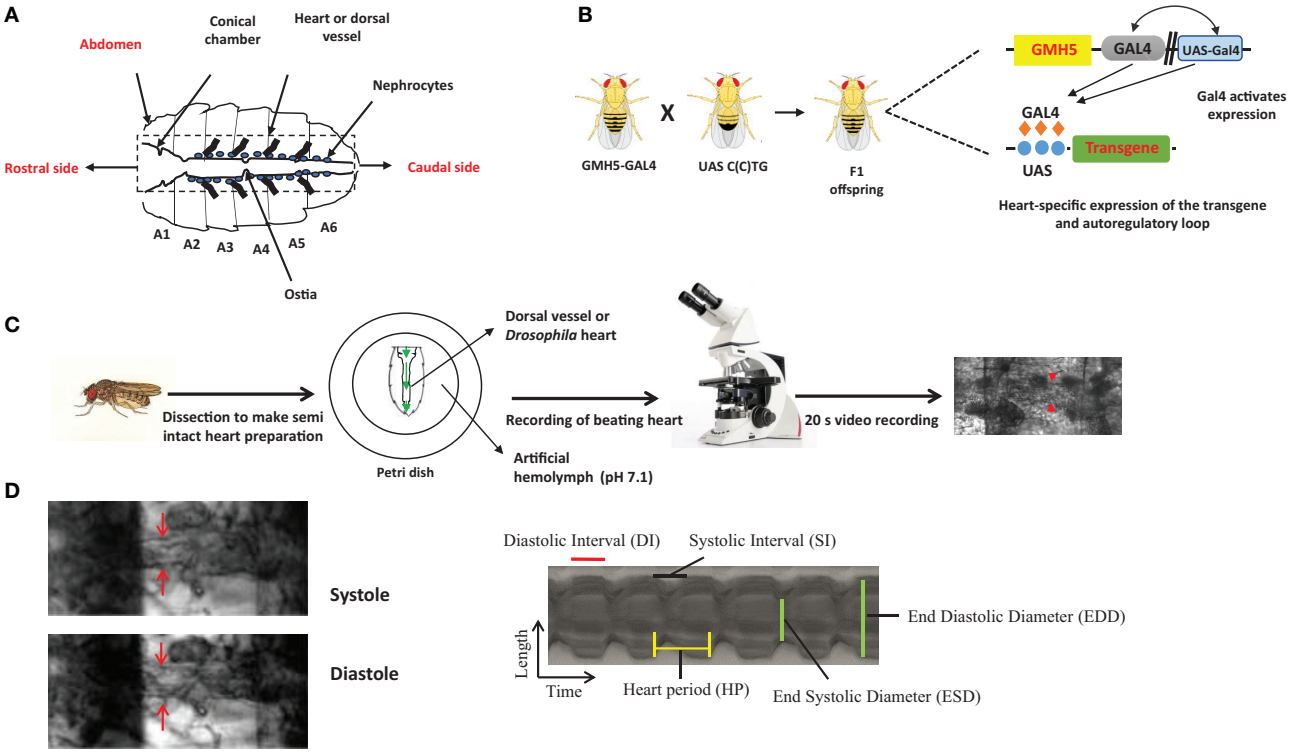


FIGURE 1 | The overall experimental set up for determination of *Drosophila* cardiac parameters. **(A)** Schematic representation of *Drosophila* heart or dorsal vessel. The heart extends from A2 to A6 abdominal segment. The conical chamber, which is present at the beginning of the dorsal vessel. The ostia which are the openings in the heart help to redistribute hemolymph from the heart to the body cavity. The bold lines near the heart represent alary muscle which connect the heart to the cuticle. The pericardial cells or nephrocytes are marked in the figure which has kidney like functions. The rostral and the caudal side of the heart are marked respectively. **(B)** Gal4-UAS system is used to express transgenes in the *Drosophila* heart. GMH5-Gal4 flies are crossed with UAS strains carrying the different length of repeats to drive the repeat expression in the F1 offspring. **(C)** F1 female flies are anesthetized, dissected and maintained in aerated artificial hemolymph solution (pH 7.1). The dissected hearts are recorded for 20 s with a high-speed digital video camera and processed with SOHA. The red arrow represents the *Drosophila* heart. **(D)** A *Drosophila* heart is marked at systole and diastole phase. A representative 2D kymograph indicates heart period (HP), end systolic and end diastolic diameters (ESD and EDD), systolic and diastolic intervals (DI and SI).

channels, instead of sodium channels, are more important to generate heart action potentials in flies. *Drosophila* has a very simple tubular-like heart structure without definitive atria or ventricle structures. However, the implementation of advanced electrophysiological techniques will help to closely describe fly heart functioning and may potentially discover additional levels of fundamental conservation between *Drosophila* and mammals.

The advantages of invertebrate genetics are utilized nowadays to study cardiac development and model diseases. Compared to mice, fly models are easy to create and maintain. In general, high mortality and low breeding rate limit the usage of mice. Chief among *Drosophila* genetic tools, is the ability to target a specific transgene expression to virtually any fly tissue and developmental time in a time frame of 6–8 weeks. This normally requires the binary Gal4-UAS system derived from yeast (59). Gal4 flies control the tissue-specific expression of yeast Gal4 transcription factor through promoters of interest and UAS flies carry specific UAS sequence upstream of the transgene of interest, which is expressed upon crossing with different Gal4-drivers. The effects of tissue-specific gene expression are observed in the progeny (**Figure 1B**). For example, in F1 offspring the Hand-Gal4 strain drives expression of the UAS-transgene to embryonic cardiogenic mesoderm (60) and tinC-Gal4 drives cardioblast-specific expression of transgenes (61).

DROSOPHILA MODELS OF CARDIAC DYSFUNCTION REPRODUCE ASPECTS OF DM1 AND DM2 PATHOLOGY

In order to model DM1 and DM2 cardiac dysfunction in flies, UAS-CTG and UAS-CCTG fly lines carrying either 250 CTG or 1100 CCTG non coding pure expansions were generated, respectively, which are within the pathological range reported in the patients (62, 63). As controls, flies carrying 20x repeats were generated. The UAS fly lines were crossed with the cardiac-specific driver GMH5–Gal4 to express the repeats in the heart. The cardiac dysfunction phenotypes of F1 flies expressing repeats in the cardiomyocytes were analyzed at several levels:

DM1-Like Molecular Alterations

At the molecular level, it has been shown that Muscleblind-like proteins are sequestered in ribonuclear foci and play

a prominent role in the disease manifestation. In control *Drosophila*, Muscleblind was not detected in the embryonic heart (64) but in adult cardiomyocytes, it is clearly detected. In the fly heart cells, Muscleblind displayed a dispersed expression throughout the nucleus and cytoplasm (25). Fluorescence *in situ* hybridization (FISH) followed by immunofluorescence technique showed that, upon long CUG or CCUG repeats expression in the cardiomyocytes, Muscleblind became sequestered into ribonuclear foci. In contrast, flies expressing a small number of either type of repeats did not show any foci or Muscleblind accumulation (25, 26).

Muscleblind sequestration leads to missplicing of several important transcripts such as CLCN1, CaV1.1channel, and IR causing different disease phenotypes such as myotonia, muscle weakness, and insulin resistance, respectively (65–67). In DM fly hearts, the inclusion of exon 13 of Serca gene and exon 16 of Fhos gene was significantly altered. These data established that Muscleblind functional depletion observed in DM1 and DM2 fly hearts is due to Muscleblind sequestration in foci (26).

It has been shown previously that expression of the long CTG repeats induces autophagy and has been proposed to cause muscle atrophy in flies (68). Among different autophagy-related genes, expression of Atg4, Atg7, and Atg12 was found to be significantly upregulated in fly muscles expressing the repeats (68). Importantly, these genes were also overexpressed in case of either long CUG or CCUG repeats expression in heart, compared to control flies expressing GFP or short repeats. These data highlighted, for the first time, a potential role for dysregulated autophagy pathway in DM cardiac dysfunction upon expression of expanded repeats (26). Nevertheless, the mechanistic connection between autophagy and heart defects in flies is still missing.

Cardiac Performance of DM Flies

Mature fly hearts were dissected in artificial hemolymph to record heart-beating with a high-speed video camera in order to study heart function (**Figure 1C**) (for a detailed description see (69)) (70). Heartbeats are analyzed using SOHA method for quantifying different parameters (71). It generates records of heart wall movement with high-resolution known as M-modes which illustrate the rhythmicity and the dynamics of the heart contractions (**Figure 1D**) (72). It allows quantification of the following parameters: relaxation and contraction phase (DI, and SI, for diastolic and systolic interval), heart period (HP), arrhythmia index (AI), end systolic diameter (ESD), end diastolic diameter (EDD) and the percentage of fractional shortening (%FS, FS = EDD – ESD/EDD × 100), which is a measure of heart's contractility (**Figure 1D**). It has been observed that expression of C(C)UG repeats in cardiomyocytes resulted in prolongation of HP. This increasement occurred via increased DI and SI. Reduction in %FS, and increased AI were also seen. SI and DI were more affected in DM2 flies than in DM1 flies (**Figure 2**). Surprisingly, short repeat expression in heart produced a significant lengthening of systolic interval and this prolongation was Muscleblind independent as foci were absent (26).

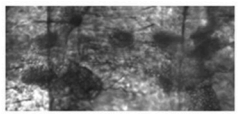
Functional Assays

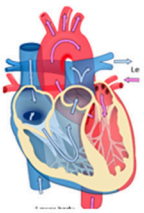
To assess the functional consequences of the expanded repeat expression, survival curves, and climbing, and flying performance tests were obtained from DM model flies. A significant reduction in survival, almost to half as compared to the control flies, was observed upon expression of expanded C(C)UG. Of note, flies expressing short repeats have similar survival to that of control flies. However, climbing velocity and flying performance of these model flies were not affected. These data suggested that reduction in the %FS of these model flies did not affect acute workload demands (flight, and climbing), but did have an important detrimental effect on life-span (26).

TESTING OF CANDIDATE THERAPEUTICS IN THE *DROSOPHILA* CARDIAC DYSFUNCTION MODEL OF DM1

To determine whether DM transgenic flies could be used as an *in vivo* tool to search for potential therapeutic compounds against DM1 cardiac dysfunction, the effect of a known anti-DM compound was tested on the DM1 *Drosophila* cardiac dysfunction model. Several small molecules that hamper the toxic Muscleblind-CUG interaction show important anti-DM1 activity (73). Pentamidine, which has been shown to inhibit the toxic Muscleblind -CUG interaction, lessen the generation of ribonuclear foci, and release Muscleblind from the foci in treated cells, rescue partially the missplicing dysfunction of two pre-mRNAs in mice expressing CUG^{exp} *in vivo* (74) were tested in DM1 fly heart models. Pentamidine, diluted in DMSO was added to the fly food at a final concentration of 1 μM (25). The effect of Daunorubicin hydrochloride was also tested in DM1 model flies. This drug was discovered in an *in vitro* fluorescence polarization screening (70). Daunorubicin, a dsDNA and dsRNA intercalant binds competitively to the CUG repeats and inhibits MBNL1 binding. It was tested in flies under the same conditions as pentamidine. The molecular and physiological parameters were compared between the model flies treated with both compounds and with the solvent only. Flies fed with the solvent had no effect on the heart performance. In pentamidine treated flies, however, heart performance was notably improved; significant reduction in arrhythmicity and an important recovery of contractility were observed. Although affected SI and DI, representative of the systolic and diastolic dysfunction as reported in patients, were not completely rescued by pentamidine (25). Compared to pentamidine, daunorubicin treatment made a remarkable improvement in the heart performance of the model flies including SI and DI (70). Importantly, the improvement of cardiac parameters was enough to recover the median survival of the flies taking both compounds.

At the molecular level, cell and mice model experiments suggest that pentamidine and related compounds might bind the CTG.CAG repeat DNA and inhibit transcription (75). However,





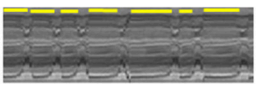
C(C)UG expression in the fly heart

C(C)UG expression in the human heart

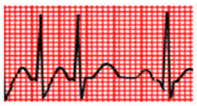
Functional Reduced median survival and life span of the flies

Sudden death in early ages due to cardiac complication

Physiological



1. Lengthening of HP
2. Arrhythmia
3. Contractility defect (%FS↓)



Lengthening of PR interval and QRS complex

1. Conduction problem
2. Arrhythmia
3. Reduction in Ejection fraction
4. Cardiomyopathy

Molecular

1. Presence of ribonuclear foci in the fly cardiomyocyte nuclei (Chakraborty et al. 2015; Cerro-Herreros et al. 2017)
2. Muscleblind found sequestered in the foci (Chakraborty et al. 2015; Cerro-Herreros et al. 2017)
3. Splicing alterations of Fhos and Serca genes (Cerro-Herreros et al. 2017)
4. Induction of the autophagy pathway (Cerro-Herreros et al. 2017)

1. Ribonuclear foci formation in the heart (Mankodi et al., 2005)
2. Muscleblind sequestration in the foci (Mankodi et al., 2005)
3. Splicing misregulation of different genes (Freyermuth F. et al., 2015)

FIGURE 2 | The physiopathological parallelisms between model fly heart and diseased human heart. Repeat expansion in both fly and patient heart causes a marked reduction in the lifespan. The expression of the repeats in the fly heart causes conduction defects, arrhythmia and contractility defects as observed in DM patients. At the molecular level, microsatellite expansion in the heart causes Muscleblind sequestration in the ribonuclear foci and this sequestration leads to misregulation in alternative splicing both in fly and human heart. Induction in the autophagy is also observed in the fly heart.

no significant difference in the transcript level was observed in flies taking both treatments compared to DMSO. In contrast, double FISH and immunofluorescence showed that ribonuclear inclusions were absent in cardiomyocyte nuclei and Muscleblind was distributed throughout the nucleus upon treatment. Taken together these data support that the compounds' effect was mediated by dispersing Muscleblind from sequestration rather than decreasing the expression level of toxic RNA. Indeed, the degree of recovery was different depending on the drug, e.g., pentamidine did not rescue the SI or DI but daunorubicin rescued both. Although speculative, it is tempting to suggest that differences in the extent of recovery may originate from a greater release of Muscleblind by daunorubicin than pentamidine.

The above results strongly suggest that Muscleblind sequestration contributes to heart dysfunction. To specifically address this question, Mbl isoform C (76) was overexpressed together with CUG repeats in *Drosophila* cardiomyocytes. Importantly, all the cardiac parameters including HP, AI, SI, and %FS significantly recovered in DM1 flies that overexpress Muscleblind, except for diastolic interval, that perhaps requires higher overexpression or presence of other Muscleblind protein isoforms (70).

CONCLUDING REMARKS

This review gives insight into the recent findings related to the development of *Drosophila* models to understand the pathophysiology of the DM cardiac dysfunction and search for therapeutic approaches. In short, expression of long CTG/CCTG repeats in the fly hearts reproduces conduction defects, arrhythmia and contractility defects observed in patients. Additionally, expanded repeats sequester Muscleblind, which significantly alters at least two alternative splicing events. Unlike in human patients, expanded CCTG repeat expression in fly heart generates cardiac phenotypes comparable to the alterations caused by CTG repeats suggesting that unknown modifiers in DM2 patients might be quenching the toxicity of repeats. The discovery of rbFox as modifier of DM2 muscle phenotypes (77) may shed some light on this question, as it may similarly dampen DM2 cardiac manifestations. Invertebrate models have proven that inhibition of Mbl sequestration in toxic RNA is also a valid strategy to treat cardiac defects in DM. However, further development of potential therapies is needed to provide a valid therapeutic candidate for treating DM cardiac features in humans.

AUTHOR CONTRIBUTIONS

MC, BL, and RA have made an equal contribution to the work, and approved it for publication.

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REVIEW
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Abnormalities in Skeletal Muscle Myogenesis, Growth, and Regeneration in Myotonic Dystrophy

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Myotonic dystrophy type 1 (DM1) and 2 (DM2) are autosomal dominant degenerative neuromuscular disorders characterized by progressive skeletal muscle weakness, atrophy, and myotonia with progeroid features. Although both DM1 and DM2 are characterized by skeletal muscle dysfunction and also share other clinical features, the diseases differ in the muscle groups that are affected. In DM1, distal muscles are mainly affected, whereas in DM2 problems are mostly found in proximal muscles. In addition, manifestation in DM1 is generally more severe, with possible congenital or childhood-onset of disease and prominent CNS involvement. DM1 and DM2 are caused by expansion of (CTG•CAG)n and (CCTG•CAGG)n repeats in the 3′ non-coding region of *DMPK* and in intron 1 of *CNBP*, respectively, and in overlapping antisense genes. This critical review will focus on the pleiotropic problems that occur during development, growth, regeneration, and aging of skeletal muscle in patients who inherited these expansions. The current best-accepted idea is that most muscle symptoms can be explained by pathomechanistic effects of repeat expansion on RNA-mediated pathways. However, aberrations in DNA replication and transcription of the DM loci or in protein translation and proteome homeostasis could also affect the control of proliferation and differentiation of muscle progenitor cells or the maintenance and physiological integrity of muscle fibers during a patient's lifetime. Here, we will discuss these molecular and cellular processes and summarize current knowledge about the role of embryonic and adult muscle-resident stem cells in growth, homeostasis, regeneration, and premature aging of healthy and diseased muscle tissue. Of particular interest is that also progenitor cells from extramuscular sources, such as pericytes and mesoangioblasts, can participate in myogenic differentiation. We will examine the potential of all these types of cells in the application of regenerative medicine for muscular dystrophies and evaluate new possibilities for their use in future therapy of DM.

Keywords: myotonic dystrophy, myogenesis, mesoangioblast, myoblast, muscle stem cell, pericyte, proteotoxicity, RNA toxicity

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INTRODUCTION

Skeletal muscle formation, growth, and maintenance in vertebrates are dynamic processes in terms of tissue differentiation, remodeling, repair, and regeneration. During the different phases of life, muscle may suffer due to injury or disease, causing weakness, pain, or paralysis, which may be even fatal. Muscle problems may be acute or short-lived, like during an infection, or be long-lasting, as

in chronic disorders. Patients with inherited myopathy or muscular dystrophy, a heterogeneous group of disorders for which disease etiology is rooted in the genetically abnormal pathways that control formation and physiological integrity of skeletal muscle, commonly experience progressive muscle weakness and atrophy (i.e., loss of muscle mass). As a result, physical strength and independence are lost, which causes substantial morbidity over decades. For the development of novel therapies to halt or reverse progression of muscle problems, validated classification criteria for differential clinical diagnosis and detailed preclinical knowledge about what is going wrong at the molecular and genetic level are a prerequisite. Unfortunately, the current states of clinical and fundamental understanding—and hence the prospects for treatment—vary enormously between individual myopathies and dystrophies.

This review is meant to bring new background knowledge for myotonic dystrophy (DM). DM is one of the most prevalent and probably also one of the most difficult to understand genetic disorders, due to its heterogeneity and its highly complex and variable clinical manifestation and molecular etiology. DM is the collective name for a disease with two genetic subtypes, DM1 (OMIM #160900) and DM2 (OMIM #602668). In fact, the classification as a skeletal muscle dystrophy is only partially correct, as the disease also has neuromuscular character and cardiac, CNS and endocrine problems are commonly involved as well (1–3). Here, we will only briefly recapitulate the history of clinical and molecular research in DM as multiple comprehensive reviews have been published on this subject (1, 2, 4, 5). The focus here is on a (re)examination of studies related to the molecular and histomorphological problems that occur during growth, maintenance, and aging of skeletal muscles in patients with DM. Findings in animal model studies are included only if they faithfully reflect the muscular pathophysiology in DM patients (6–8).

The main waves of myogenesis occur during embryonic development and growth, when myoblasts undergo cell cycle arrest and fuse to form the multinucleated myotubes that ultimately become the mature myofibers (9–11). Later, regenerative myogenesis serves in muscle turnover and to replace damaged or diseased muscle (10). Relevance of embryonic and adult stem cells for each of the distinct phases of myogenesis for the manifestation of DM will be examined. We will also describe so-called non-somite skeletal myogenesis through involvement of mesoangioblasts (MABs) and pericytes (PCs) as muscle progenitor cells, and speculate about the importance of this process for DM. Finally, we will discuss possibilities to use these progenitor cells in future therapeutic strategies.

MYOTONIC DYSTROPHY

Clinical Features and Genetic Causes

A number of clinical and molecular characteristics are shared between DM1 and DM2, but the differences prevail and render them distinct disorders.

Myotonic Dystrophy Type 1

Myotonic dystrophy type 1, or Steinert's disease, shows the highest prevalence, ranging between 0.5 and 18 cases per 100,000 individuals among different ethnic populations (12–14). Progressive muscle weakness and atrophy of the distal muscles together with myotonia are consistent features. Multiple other organs in the body can also be affected, causing combinations of symptoms. For example, heart failure due to conduction problems, insulin resistance, excessive sleepiness, intellectual disability or mental problems, and cognitive deficits are common symptoms (15–18). Anticipation is typical for DM1, which means that disease problems become more severe and occur earlier in successive generations in families. Nowadays, five partially overlapping clinical subtypes of DM1 are recognized, based on the occurrence and onset of the main symptoms: congenital (cDM), infantile, juvenile, adult, and late-onset/asymptomatic DM1 (19). This classification is not only important for patient care but also for the design of clinical trials (2). For a fair interpretation of the literature cited in this review, it is important to note that in studies that appeared before the recent redefinition and refinement of disease classes, authors mostly only discriminated between cDM and adult-onset DM1.

The sole known molecular cause of DM1 is the expansion of a (CTG•CAG)n sequence on chromosome 19q13 in the last exon of *DMPK* (20, 21) (**Figure 1**). In DM1 families, when expanded to a length above (CTG)37, the repeat is unstable and has a tendency to grow somatically and intergenerationally (22, 23). Thus, repeat expansion forms the basis for the anticipation phenotype, whereby a longer repeat correlates with more severe symptoms and an earlier disease onset. An expanded *DMPK* repeat is mostly an uninterrupted (CTG)n sequence of variable length. However, additional sequence variations such as CCG and CGG triplets in the 3′ end or immediate flanking DNA, or non-CTG replacements within the repeat have been found. These alterations are generally associated with milder disease manifestation and symptomatic variation in families or seem to occur somatically in certain tissues (24–26).

From the normal and mutant *DMPK* alleles multiple alternatively spliced transcripts are produced, all of which contain the (CUG)n repeat sequence in their 3′ untranslated region (UTR) (27). In addition, there is a partial overlap with an antisense-oriented gene, named *DM1-AS*, which encodes variant (CAG)n transcripts with characteristics of long non-coding RNA (lncRNA) (28).

Myotonic Dystrophy Type 2

Formerly known as proximal myotonic myopathy and proximal myotonic dystrophy, DM2 was discovered in a group of patients with clinical features that were slightly different from those in DM1 (29, 30). Prevalence for DM2 varies strongly by population, but is less well known than for DM1, since the mild DM2 phenotype often goes undiagnosed (5). As mutations have been predominantly identified in Caucasians in Northern Europe and this population also has the most registered DM2 patients (31, 32), prevalence of DM2 and DM1 may be quite similar in countries in this region (33). Although the myotonic dystrophies share a number of clinical symptoms, there are distinct differences (34, 35) (**Table 1**). For DM2 no congenital manifestation is known and diagnosis is always late, when patients have reached adult age. Myotonia is less evident and myotonia of grip often

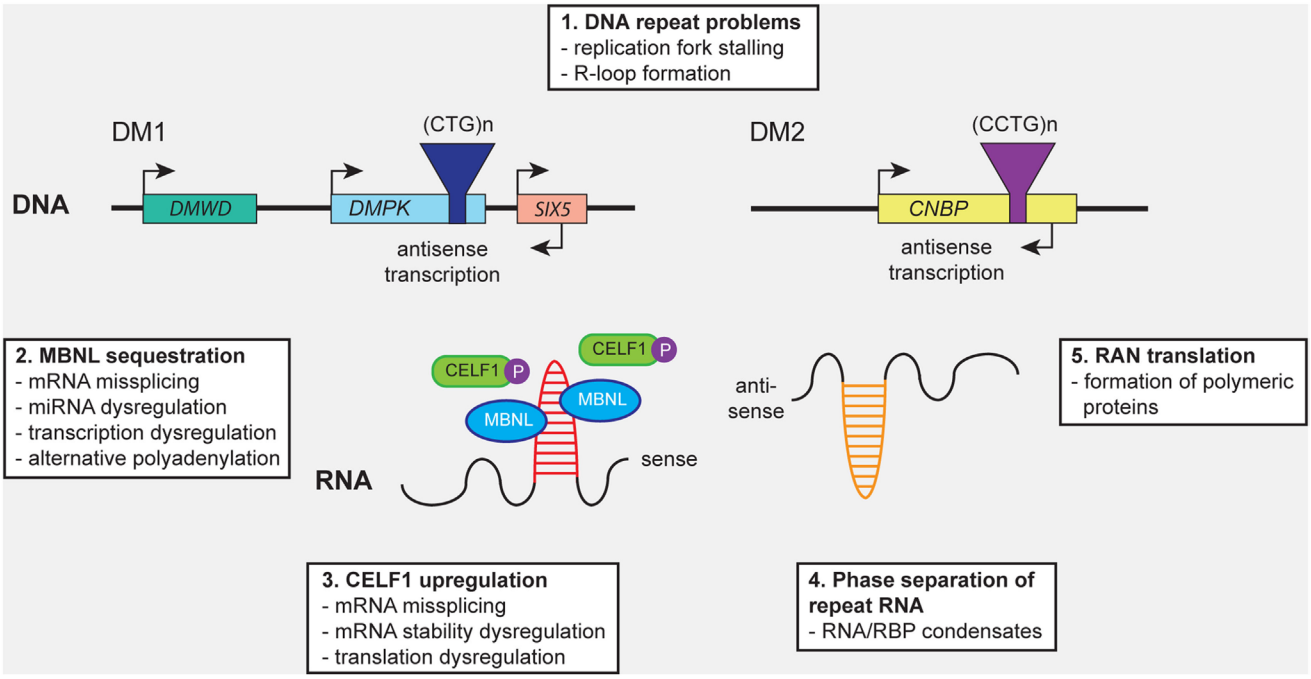

FIGURE 1 | Distinct molecular mechanisms contribute to pathology in myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2). (1) Expanded (CTG)n and (CCTG)n repeats in *DMPK* and *CNBP*, or the complementary repeats in the antisense genes (not shown), can cause cellular stress by (1) promoting DNA replication fork stalling and R-loop formation. Expression of repeat-containing sense and antisense RNAs results in (2) sequestration of members of the MBNL protein family, leading to mRNA missplicing, alternative polyadenylation, microRNA (miRNA) deregulation, and transcription deregulation. In addition, (3) CELF1 gets hyperphosphorylated and stabilized, resulting in mRNA missplicing and dysregulation of mRNA stability and translation. (4) Formation of abnormal RNA-protein condensates by repeat RNA and RNA-binding proteins (RBPs) may alter the intracellular distribution fate and biological activity of RBPs. (5) Repeat-associated non-ATG (RAN) translation of the repeats may result in the production of toxic polymeric polypeptides, which perturb cellular proteostasis.

TABLE 1 | Similarities and differences in genetic, clinical, and histopathological features of myotonic dystrophy type 1 (DM1) and myotonic dystrophy type 2 (DM2).

	DM1	DM2	Reference
Main features			
Affected gene, chromosome	*DMPK*; 19q13.3	*CNBP*; 3q21	(20, 32)
Repeat expansion	(CTG)n	(CCTG)n	(20, 37)
Anticipation	Always present	Exceptional	(38)
Age of onset	Any age	Adulthood	(19)
Congenital form	Yes	No	(19)
Muscle symptoms			
Predominant muscle weakness	Distal	Proximal	(39)
Predominantly affected muscle fibers	Type 1	Type 2	(40–42)
Histopathological findings			
Fiber atrophy	Type 1 fibers (not always present)	Subgroup of highly atrophic type 2 fibers (always present)	(30)
Nuclear clump fibers	In end stage only	Scattered at early stage	(43)
Sarcoplasmic masses	Frequent in distal muscles	Extremely rare	(43)
Ring fibers	Frequent	May occur	(43)
Internal nuclei	Massive in distal muscle	Variable, mainly in type 2 fibers	(43)

has a jerky quality (36). Proximal muscles are most prominently affected in DM2 and weakness and wasting of facial muscles and limbs is generally mild (29, 30, 36).

Similar to DM1, only one underlying cause of disease has been identified for DM2: all patients carry an expansion of a (CCTG)n repeat in intron 1 of *CNBP* (previously known as *ZNF9*) on chromosome 3q21 (29, 30) (**Figure 1**). The repeat is part of a complex (TG)n(TCTG)n(CCTG)n motif in which the (CCTG)n repeat is often interrupted and consists of up to 26 units in healthy individuals. In patients the (CCTG)n repeat is usually uninterrupted and contains 75–11,000 quadruplets (36). The DM2 repeat is extremely unstable and has a tendency to expand somatically, causing length increase and cell-to-cell heterogeneity during a patient's life. Interestingly, by contrast to the behavior of the (CTG•CAG)n repeat in DM1, the (CCTG•CAGG)n repeat has the tendency to contract intergenerationally (44). The correlation between repeat

length and disease severity is less strong than in DM1 patients and anticipation is less evident (1, 38).

Molecular Mechanisms Involved in the Etiology of DM1 and DM2

Several molecular mechanisms are thought to contribute to the muscular pathogenesis of DM throughout all phases of development and maintenance (**Figure 1**). Similarities with other neurological disorders that are caused by microsatellite expansions have already been comprehensively reviewed (4, 8, 18, 45, 46). Here, we aim to accentuate the relationships between the molecular and cellular levels at which problems caused by the repeat expansions may occur. The emphasis is biased toward the pathobiology of DM1, based on a longer history of study, its seemingly bigger variability and complexity of manifestation, and the broader availability of patient materials, and cell and animal models.

Problems at the Chromatin Level

The first level at which repeat expansion may contribute to disease is at the chromatin level. The (CTG•CAG)n repeat in DM1 is situated within the 3′ UTR of *DMPK*, within the overlapping antisense *DM1-AS* gene and in the promoter of *SIX5* (formerly known as *DMAHP*). These genes lie in the center of a gene-rich region of chromosome 19, spanning also *DMWD* (47, 48), *RSHL1*, and *SYMPLEKIN,* within a chromatin loop that is flanked by nuclear matrix attachment regions (49). Two binding sites for the transcriptional repressor CTCF with an insulator role in regulation of transcription and chromatin architecture are within this loop, flanking the repeat area. Already soon after the discovery of the repeat, Otten and Tapscott demonstrated that long (CTG•CAG)n repeats are strong nucleosome positioning elements (50). Extreme repeat expansion as in cDM leads to the occlusion of adjacent DNase hypersensitive sites and concomitant changes in local DNA methylation in the surrounding CG-rich region (51–53), rendering the chromatin more heterochromatic and inaccessible. In turn, this process has *cis*-effects on gene activity in the immediate vicinity, including *DMPK*, *DM1-AS*, and *SIX5* and perhaps other neighboring genes. To our knowledge, no similar studies of epigenetic changes after repeat expansion in *CNBP* (DM2) exist. Clearly, more work is needed to understand the biological effects that DNA methylation, histone modification and other chromatin changes due to repeat expansion in the DM1 locus have on muscle progenitor cells.

Problems at the DNA Level: Stalled Replication Forks and R-Loops

Numerous studies have addressed DNA instability of expanded (CTG•CAG)n and (CCTG•CAGG)n repeats. The influence of oxidative damage and mismatch-repair and recombination pathways for DNA repair on repeat instability have already been thoroughly discussed (54–56). Less attention has been focused on the types of cell stress that large repeats may have at the DNA level and their consequences for loss of cell viability.

DNA polymerase stalling and replication fork arrest seem to be frequent events when unusually large repeat sequences in the genome have to be replicated in S-phase (57). Cells have adequate repair systems to resolve problems with DNA replication fork processivity, either directly when proceeding through the cell cycle or later when they arrive at so-called DNA replication checkpoints (58). Different rescue systems exist in which Chk1 and γH2AX phosphorylation and p53 activation are crucial for the on-site response (58). Stalling at sites in eu- and heterochromatin may even require differential composition of the repair machinery that is recruited. For transcribed repeats, as in the DM1 and DM2 loci, there is an additional complication. Here the threat comes from the formation of so-called R-loops (59). R-loops are triple-stranded RNA-DNA structures formed by duplex formation between the template strand and the transcribed RNA, leaving the non-template strand unpaired. R-loop formation may influence DNA methylation and transcriptional activity in its immediate vicinity. Persistent presence of unresolved R-loops or structures wherein stalled DNA forks and R-loops coincide may affect cellular fitness and arrest the cell cycle. The associated stress may even cause cell death.

An elegant study indeed showed that transcription of a (CTG•CAG)n repeat, as in the DM1 locus, may cause convergent repeat instability and apoptosis (60). Against this background, it is tempting to speculate that proliferating cells in which *DMPK* and/or *DM1-AS* are expressed are vulnerable to the danger of formation of stalled replication forks and R-loops. Specifically, this holds for all mesodermal derivatives and embryonic and adult muscle stem cells [muscle-resident stem cells (MuSCs); see below]. An identical pathogenic cascade may be possible in DM2, since *CNBP* is most highly expressed in muscle (61). There is evidence for bidirectional transcription across the locus (62) and unpaired ($CC^{T}/_{U}G$)n or (CAGG)n repeats may form abnormal hairpin structures (63).

Misregulation of RNA Processing and Translation

By far the most intensely studied aspects of DM's etiology are the pleiotropic problems caused by the production of repeat-expanded transcripts. Intranuclear residence of repeat transcripts causes *trans* effects, which culminate in abnormal processing of many other RNAs in the cell's transcriptome (64).

Probably right after transcription, the repeats in RNAs of *DMPK* and *CNBP* (and the corresponding antisense genes) form stable hairpins that alter activities of two antagonistic protein families, the MBNL (Muscleblind) and CELF proteins. MBNL proteins bind anomalously across the repeat hairpin, leading them to become sequestered in nuclear aggregates, which are visualized as so-called foci under the microscope (65–71). Various other RNA-binding proteins (RBPs) such as hnRNP F, H, DDX5, -6, -17, and Staufen, some of which have intrinsically unstructured domains, are engaged in the nuclear aggregates as well (71–74). CELF1, formerly called CUGBP1, binds at the base of the hairpin and becomes hyperphosphorylated.

Altogether, these events result in an imbalance in cellular ribostasis and proteostasis, associated with depletion and a shift in the distribution of MBNL family members and an increase and redistribution of CELF1 protein. The end result is a cell type and cell state dependent whole-transcriptome effect on alternative splicing (75–78), alternative polyadenylation (79, 80), and nucleocytoplasmic transport of other transcripts for which

MBNL1-3 or CELF1 play a role in RNA processing. Changes in mRNA half-life may also occur, as CELF1 has been identified as a key regulator of RNA decay or translational silencing in muscle cells (81). In turn, the changes in the transcriptome have widespread *trans*-acting effects on the production and makeup of multiple proteins (82–86). Some cell-stage effects of MBNL1-3, CELF1, and other ribonucleoprotein (RNP) anomalies will be discussed in more detail below, in the context of embryonic or regenerative myogenesis.

Missplicing may have the most obvious links with the myopathy in DM. For instance, abnormal splicing of *ClC1* is sufficient to cause myotonia (87). Missplicing of the muscle-specific genes *BIN1, TNNT3*, *RYR1*, *TTN*, *LDB3*, and *SERCA1* is linked to impaired muscle function (88). Aberrant splicing of the insulin receptor, highly expressed in skeletal muscle, results in reduced responsiveness to insulin, another contributing factor to skeletal muscle dysfunction (89–91). Furthermore, alternative splicing of *CACNA1S*, a calcium channel that controls skeletal muscle excitation-contraction coupling, is markedly repressed in DM1 and DM2 (92). Combined with splicing alterations in the machineries for voltage-induced Ca^{2+} release and for release and uptake of Ca^{2+} in the ER/SR store (*RyR1* and *SERCA1*), this may lead to chronic Ca^{2+} overload, activate ER stress (93), or become a cause of excitotoxicity. These long-term physiological abnormalities may induce premature senescence and contribute to muscle degeneration in DM.

Not all splicing abnormalities are congruent in DM1 and DM2 muscles. For instance, *TNNT3* is more often misspliced in DM2 than in DM1, and *NCAM1* missplicing can be found more in nuclear clump fibers of DM2 patients (1, 94, 95). Furthermore, in muscle tissue of DM2 patients, *NEDD4* was found to be disrupted. *NEDD4* is an E3 ubiquitin ligase for PTEN, an important regulator of the *AKT* signaling pathway for protection against cellular stress. The PTEN protein level is upregulated in DM2 muscle tissue and PTEN accumulations can be found in nuclear clump 2a fibers in DM2 muscle (96).

For DM2, there may be also a direct effect on ribostasis and proteostasis. Repeat expansion in *CNBP* may cause pausing of transcription or retardation of splicing of its pre-mRNA, resulting in a reduction of mature *CNBP* mRNA and the CNBP protein product. Initial studies on this topic yielded conflicting results, as some groups found unaltered levels of *CNBP* RNA and protein levels in cells and tissues from DM2 patients, whereas later studies showed a clear inhibitory effect of an expanded (CCTG)n repeat (97). Studies on heterozygous knockout mice for *CNBP* brought further support for the idea that haploinsufficiency may be involved in myopathy in DM2 (98). The CNBP protein has a role in the regulation of translation through binding to the 5′ UTRs of terminal oligopyrimidine tract mRNAs. For example, the production of RPS17, poly(A)-binding protein 1, and elongation factors eEF1A and eEF2 are controlled by this mechanism (99).

Also other types of problems at the translational level may play a role in the distinct manifestation of DM1 and DM2. Differential involvement of CELF1 may herein be a key issue. CELF1 can act by relieving secondary structures on a subset of target RNAs that exhibit G-rich sequence stretches with a high-degree of secondary structure, thereby promoting their translatability. Furthermore, if (hyper)phosphorylated, CELF1 may form a multisubunit complex with eukaryotic initiation factor eIF2 and other translation initiation factors, promoting the translation of protein products from alternative start codons in mRNAs that bear an IRES motif (100, 101). Importantly, the different effects of CELF1 on the translation of target mRNAs depend on its phosphorylation status and on the overall level of available protein, which is controlled in accordance with the stage of myogenic differentiation. Although there is no consensus about the fate of CELF1 in DM1 and DM2 muscles, evidence points to a situation in which the available level and thus binding of CELF1 to mRNAs is reduced in DM2. By contrast to the situation in DM1, its phosphorylation status appears unaltered in DM2. When taken combined, these studies support the idea that, superimposed on aberrancies in RNA splicing and polyadenylation, aberrancies in protein translation might have distinct roles in eliciting muscle dysfunction in both forms of DM (99, 102).

RNP Condensates: Is Phase Separation of Repeat RNA Causing Cell Stress?

Revolutionary work on polymer physical properties of macromolecular assemblies that undergo liquid-to-gel phase transition and concentration into microscale structures have led to the idea that formation of abnormal condensates by repeat transcripts and RBPs may also be involved in repeat RNA toxicity in DM (103–105). Jain and Vale have recently provided evidence that poly-CUG RNA and also poly-CAG RNA, which both can engage in multivalent intra- and intermolecular reactions, can undergo phase separation *in vitro* (106). They also showed that (CUG)n RNA forms small phase-separated gel inclusions in cells.

More basic studies into the thermodynamics of phase transition have revealed that the threshold concentration at which nano-sized biomolecular RNP condensates are formed are determined by various parameters, including the type, stoichiometry and local concentration of available RNA, and protein constituents and their folding or solubility properties. Most of these studies have been focused on phase transition under conditions with high concentrations of RNA and protein. Future research must thus reveal the requirements for RNA-protein condensate assembly and phase transition in patient cells with endogenous levels of expanded RNAs. Most importantly, the question must be answered whether the occurrence of abnormal repeat RNP gel inclusions containing *DMPK*, *DM1-AS*, or *CNBP* mRNA with abnormal repeat length could by itself be a trigger for stress.

Repeat-Associated Non-ATG (RAN) Translation

Since its discovery in 2011, RAN translation has been linked to proteome abnormalities in multiple repeat-expansion disorders (107). RAN translation of expanded triplet or quadruplet repeats can occur in all reading frames, resulting in the production of homopolymeric (DM1) or poly-tetrapeptide (DM2) proteins (62, 108, 109). In DM1, polyglutamine nuclear aggregates have been identified in myoblasts, skeletal muscle and peripheral blood leukocytes of patients, and in DM1 mouse tissue (108). In DM2, RAN translation across the (CCUG)n and antisense (CAGG)n repeats produces toxic poly-LPAC in neurons, astrocytes, and glia cells, while poly-QAGR proteins accumulate in white matter (62).

Whether these findings can be extrapolated to DM2 muscle is an open question.

Many other unanswered questions remain about the production and relevance of RAN products in DM. How does an intronic RNA segment that is normally retained in the nucleoplasm and—without repeat gets quickly degraded—become accessible for the ribosome machinery? A similar question can be asked for DM1, since also expanded *DMPK* and *DM1-AS* RNAs are mainly retained within the nucleus, unavailable for assembly of ribosomes and subsequent translation (28). Nuclear translation is a process that has been demonstrated to occur (110, 111), but at this moment we do not know whether this could be involved. Another possibility is that the initiation of RAN translation occurs only after the onset of prometaphase in cycling cells, so when ribosome subunits are accessible because nucleoplasm and cytosol can mix. Indeed, at mitotic entry, cap-independent translation acquires a dominant role in expression regulation (112). Once polymeric proteins have been produced by RAN translation, they may—alike prion proteins—have a seeding effect in triggering abnormal protein aggregation and condensation and cause imbalance in the cellular proteome (62, 107). This may come at a considerable fitness cost for the cell in which it occurs.

Cellular Mechanisms Involved in the Etiology of DM1 and DM2

Quantitative and Qualitative Aspects Do Matter

Any of the molecular disease pathways discussed above could contribute to the myopathy during the different phases of life of patients with DM (**Figure 2**). However, one should realize that their involvement at the cellular level may differ dramatically with the stage of development and with the type of myofiber that is formed during muscle growth, regeneration, and aging. For example, stalling of replication forks at the (CTG•CAG)n and (CCTG•CAGG)n repeats may not be major threats in quiescent cells, but danger may increase once cells start proliferating. Similarly, reciprocal coupling does exist between the stage and type of differentiation and the mode and extend of alternative splicing in individual muscle progenitor cells or myofibers. The level of *DMPK* and *CNBP* transcripts, splicing factors, or their mRNA targets do, however, vary during muscle differentiation and maturation. So in muscle cells from cDM, DM1, or DM2 patients the complex changes in stoichiometric ratios between MBNL1-3, CELF1, and other RBPs, and the *DMPK* or *CNBP* RNA molecules that take place during natural development are superimposed by variable toxic changes caused by abnormal RBP-repeat RNA interactions (**Figure 1**).

New supportive evidence for a mutual relationship between differentiation abnormalities and repeat expansion effects was obtained by our group in a study of isogenic CRISPR/Cas9-edited DM1 muscle cells with and without (CTG•CAG)2600 repeat (113). Monitoring of the molecular causes and cellular effect at the individual cell level, during *in vitro* myocyte–myotube differentiation and maturation in culture should thus become possible. Answering the chicken-egg question whether the impaired differentiation and regeneration events or the RNA processing abnormalities and associated cell stress were first in initiating the pathology in DM muscle tissue is not easy. Heterogeneity in cell type composition and developmental stage in the muscle cell population is here the confounding factor. In the next sections, we will try to provide background information on aspects of normal myogenesis and the cellular pathology and histopathology of DM muscle, to come closer to the root of this problem.

Muscle Fiber Type and Developmental-Stage Dependent Manifestation of Disease

Within human skeletal muscle there are different categories of fiber types, defined by myosin heavy chain (MyHC) isoform expression and metabolic activity (114). Individual fibers are characterized as one type of slow-twitch fiber (type 1) and three types of fast-twitch fibers [type 2a, 2c, and 2x/d (also referred to as 2b)] (115). Type 1 and 2a fibers are oxidative, whereas type 2c and 2x/d fibers are primarily glycolytic. Type 2 fibers generally produce higher forces and fatigue more quickly than type 1 fibers (116). Walled off from the main part of the muscle in the muscle

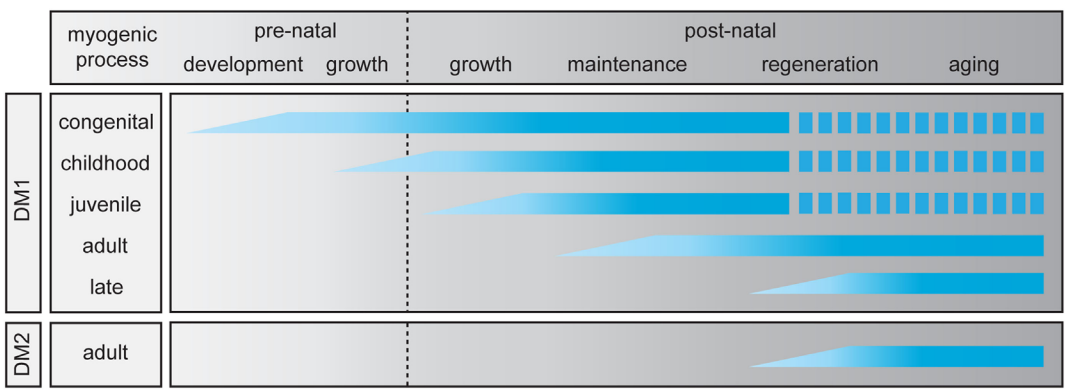

FIGURE 2 | Abnormalities in skeletal muscle myogenesis in myotonic dystrophy (DM). For myotonic dystrophy type 1 (DM1), five clinical subtypes have been identified (19), while for myotonic dystrophy type 2 (DM2) only the adult-onset manifestation is known. The myogenic process in skeletal muscle is divided in two prenatal and four postnatal stages. The graphic summarizes which stages of the pre- and postnatal myogenic process are affected in each clinical DM (sub)type. A discontinuous bar indicates decreased life expectancy.

spindle, highly specialized fibers, known as intrafusal fibers, can be found. These fibers serve as specialized stretch receptors that allow the perception and coordination of limb movement.

Most muscles in the human body are built as a mixture of type 1 and 2 fibers, but between individuals there are marked differences in muscle composition and size. Fiber type content and distribution is thereby coupled to aspects of physical performance, such as endurance and strength. Hence, there is also differential association with disease risk or states between individuals, as skeletal muscle fiber subtypes respond differently to (patho)physiological signals, which include atrophy signals. The ratio of type 1 and 2 fibers within a muscle is altered in muscular disorders when atrophy of one of the two types occurs. Several signaling pathways for muscle atrophy are known, mostly related to abnormalities in protein degradation (117). However, the selectivity of fiber type atrophy remains an unresolved issue (118, 119). For DM, the fiber type specificity of manifestation is a topic that deserves new attention, especially since revolutionary methodologies for transcriptome, proteome, and microscopy analyses at the single cell level have become available.

Skeletal muscles from all DM patients have a distinct histopathological phenotype, but biopsies show conspicuous differences between DM1 and DM2 patients (**Table 1**). The distal muscles mainly affected in adult DM1 show predominant loss of type 1 fibers (120), whereas the predominantly affected proximal muscles in DM2 show mostly type 2 fiber atrophy (39). Furthermore, an increased variation of fiber diameter and prominent central nuclei with chromatin clumps are present in DM1, normally observed in constantly regenerating muscle with immature fibers (39, 43, 121, 122). Another differential observation is the higher frequency of nuclear clump fibers in DM2. Nuclear clump fibers are typically observed in denervated muscles and have been termed "denervation-like" when observed in DM2 muscle, since other neuropathic alterations were not detected (123). Generally, the alterations seen in muscle of DM2 patients are rather mild and have a heterogeneous character (122). Muscle pathology in DM1 patients has a more typical appearance. However, histological reports, especially of older DM1 studies, may sometimes have a misleading message as researchers usually only draw a distinction between muscles of individuals with cDM and the adult-onset form of disease. Details about graded differences in pathology between muscles from patients with childhood, juvenile, adult, and late-onset/asymptomatic DM1 are not well known.

Already early on it was recognized that cDM is associated with a much broader spectrum of morpho-anatomical muscle problems, with type 1 fiber preponderance and hypotrophy and common occurrence of type 2b fiber deficiency (52, 124). Undifferentiated thin fibers and an increase in satellite cells at birth indicate immature muscle fiber growth and delayed muscle fiber differentiation (125, 126). Also, outside the body in *in vitro* culture the differentiation and maturation capacity of progenitor cells from embryonic muscle of cDM patients was found to be defective (127). The percentage of myoblasts fusing to form myotubes was reduced, the myotube morphology was abnormal, and only immature MyHC protein isoforms were expressed, primarily the embryonic isoform. Also conspicuous aberrancies in intrafusal fiber and muscle spindle presence or morphology were reported. These latter features and the specific fiber type effects may point to additional abnormalities in innervation, motor unit formation, or neurotrophic signaling during the later phases of embryonic development and early prenatal muscle maturation (128–130) (**Figure 2**).

HOW HEALTHY AND DM MUSCLES ARE BUILT AND MAINTAINED

Myogenesis During Early Embryogenesis

The skeletal muscles of limb and torso and head muscles in vertebrates derive from the paraxial and prechordal mesoderm layers in the early embryo. The myogenic process starts when the paraxial mesoderm forms multiple somites, which then further specialize and form the dermomyotome. First, a large proportion of stem cells in the somites and later in the forming limb buds undergo frequent mitosis, under influence of factors such as IGF-1 and PDGF. The proliferating progenitor cells derived from the embryonic mesenchyme of the somite then undergo different phases of myotome development. This process starts with programmed maturation accompanied by adoption of skeletal muscle fate and withdrawal from the cell cycle, giving rise to a layer of non-proliferating myoblasts that form the primary myotome beneath the dermomyotome (131, 132). When more and more cells are progressively added and start to fuse to already committed myoblasts (myocytes) that already reside in the myotome this leads to the formation of the first myofibers and the onset of embryonic muscle growth (133). The following sections will describe the different steps on the road to maturation of skeletal muscles before and after birth.

Cell Cycle Exit During Myogenesis

During all stages of contribution to muscle formation and regeneration, myoblasts first need to stop their proliferation process by exiting the cell cycle (134). This occurs by activation of cyclin-dependent kinase (cdk) inhibitor p21 and retinoblastoma protein (Rb), a downstream target of p21. p21 is also partially responsible for the decreased Cdk1 activity observed in differentiating cells (135–137). Formation of Rb–E2F complexes is necessary for maintenance of inhibition of cell cycle progression and for cell cycle withdrawal (138). The role of CELF1 in this regulatory circuit is considered an important link to myogenic problems in DM.

Phosphorylation of CELF1 regulates its intracellular localization and activity. Normally, CELF1 is phosphorylated by AKT and cyclin D3/cdk4 at Ser28 and Ser302, respectively. This post-translational modification is crucial for myogenic progression. Induction of AKT activity is otherwise involved in the suppression of apoptosis during myogenesis (139). In DM1 myoblasts, CELF1 appears to become hyperphosphorylated by AKT (140), whereas in myotubes, CELF1 phosphorylation by cyclin D3/cdk4 seems to be reduced (141). Alterations in the activity of GSK3β influence the activity in the cyclin D3-CDK4 phosphorylation signaling pathway from upstream. The abnormalities in phosphorylation status compromise CELF1's role as a translational regulator of a specific population of mRNAs. As an end effect, the changes lead

to an increase of cyclin D1, an important regulator of proliferation of myoblasts, and to a reduction of p21 in DM1 myotubes. Together, the changes in the AKT-CELF1-cyclin D1 and cyclin D3/cdk4-CELF1-p21 pathways affect the myogenic process in DM1 (68, 141, 142). Also the Rb–E2F repressor complex appears not to be formed, underscoring that impairment of cell cycle withdrawal may have a role in both forms of DM manifestation (68). However, because not all pathways in which CELF1 is involved are similarly abnormal in DM1 and DM2, other obstructions in myogenic programming might be at play in DM2 as well.

Myoblast Fusion

After cell cycle arrest, the fusion of competent myoblasts to form multinucleated myotubes begins. Fusion is a tightly controlled process that involves distinct mechanistic steps, including cell–cell interaction, recognition, and adhesion, followed by membrane coalescence and merging of competent myoblasts to form the multinucleated myotube (143). Extracellular signals from adjacent tissues have an important role in the initiation of several of these steps. Two waves of fusion events take place to form the muscle. Primary myofibers that determine the shape and identity of muscles are formed in the first wave. Secondary myofibers align alongside the primary myofibers and add mass to the muscles in the second wave. Distinct events govern these stages for promotion of differentiation and growth of muscle: first, individual myoblasts fuse to form nascent myotubes and then multinuclear myotubes are formed during subsequent fusion steps between myotubes and additional individual myoblasts (144–146).

The factors that trigger cell fusion (i.e., fusogens) are not precisely known, but numerous proteins that coordinate the formation of primary and secondary myotubes have been identified (144, 146–148). Myomaker, a plasma membrane, Golgi and organellar membrane embedded protein seems crucial (149, 150). Its importance is illustrated by the finding that mutations in myomaker cause a congenital myopathy, Carey-Fineman-Ziter syndrome (151, 152). Other proteins that have an essential role in the myoblast fusion process are myomixer and myomerger. Myomixer, localized to the plasma membrane, associates with myomaker. Myomixer together with myomaker are strong promoters of cell fusion, driving the formation of multinucleated cells from myoblasts (153). Myomerger is only expressed on myocytes and induces the fusogenicity, while myomaker is essential to make a cell fusion competent (148).

Rearrangements in the actin cytoskeleton are first involved in the formation of membrane protrusions between the incoming myoblast and the partner myoblast or myotube. Later they are important for pore formation and cytoarchitectural rearrangements in the resulting multinuclear cell. The entire network that controls the actin network in cells is too complex to discuss here (154, 155), but one issue related to DMPK splice variants may be important. Tentative evidence points to a role for the kinase activity of DMPK, a member of the Rho kinase family, in the regulation of myosin light chain phosphorylation. DMPK may, therefore, functionally link to plasticity of the actomyosin network (156, 157). DMPK is dispensable for myogenesis, as *DMPK* knockout mice are viable and make muscles with only minor abnormalities (156). However, the possibility that *DMPK* splicing becomes spatiotemporally deranged by presence of very long (CUG)n repeats and exerts a modulatory effect on actomyosin cytoskeleton dynamics during early and late myoblast-myotube fusion still exists. Tight regulation of DMPK isoform E during early muscle differentiation is essential for normal development (158) and alternative splicing causes downregulation of DMPK E during myoblast to myotube differentiation (159).

Generally, the muscle problems in adult DM patients are difficult to attribute to any of the distinct phases that determine the differentiation, fusion, or senescence or death of different types of muscle cells *in vivo*. *In vitro* studies on myoblast cultures of adult-onset DM1 with intermediate expansions or DM2 patients are scarce. New methodology was recently published for the immortalization of primary satellite cells, which stimulate *in vitro* studies of differentiation capacity (86). Interestingly, DM2 satellite cells with (CCTG•CAGG)4000 repeats did not have a significantly altered myogenic capacity, confirming earlier findings (66, 160). By contrast, more attention has been concentrated on the study of embryonic or early postnatally derived muscle progenitor cells from cDM muscle. These cells consistently showed impaired myogenic potential and reduced myogenic differentiation capacity during culture *in vitro* (66, 127, 160–164).

Transcription Factor-Induced Programming of Myogenic Lineages

To better understand pathological changes in muscle in DM patients, we will first examine the molecular processes that govern normal muscle development (165–168) and discuss these against the background of repeat expansion. The molecular cascade that directs the fate of somite-derived cells during developmental maturation is principally determined by *PAX3* and *PAX7*. These transcription factors trigger the sequential expression of a group of highly conserved myogenic regulatory factors, collectively known as MRFs. MRFs contain a basic helix-loop-helix domain and recognize the E-box in the promoter of target genes (169). *MYF5* and *MYF6* (also known as *MRF4*) act as upstream regulators of *MYOD,* perhaps the best-known member of the family. Co-expression of these three factors is required for myogenic commitment. Then a fourth factor, myogenin (*MYOG*) activates advancement to the myocyte stage and terminal differentiation of the muscle cell (166–168, 170). In this circuit, myogenic transcription factors act in a complex feedback and feedforward network. For instance, the temporal coordination of *MRF*-mediated gene expression is achieved by allowing certain genes to be directly activated by an individual MRF, whereas the induction of other genes in later stages of differentiation by the same MRF requires the participation of the earlier target gene products (166). There is compelling evidence that the expression of various proteins in this MRF regulatory network, like MYOD and MYOG, is affected by the expansions in DM1 or DM2 (69). The involvement of RBPs is thereby a key event. CELF1, for example, binds and destabilizes *MYOD* mRNA *via* its GRE-motif, and an increase in CELF1 activity thus has an inhibiting effect on the progress of myogenic differentiation (72).

Members of the *SIX* family of homeobox genes (*SIX1, SIX2, SIX4*, and *SIX5*) are among the other upstream regulators of MRFs. In mice, *Six4* and *Six5* repress *Myog*, whereas *Six1* activates

it (171). *Six1* and *Six4* were shown to be required for *Pax3* and *MRF* expression during myogenesis (172). Interestingly, *SIX5* is immediately adjacent to *DMPK* and its mRNA level seems decreased in DM1 patients (173). *Six5* knockout mice, however, show essentially no muscle symptoms. Hence, the role and relevance of *Six5* in DM1 muscle pathology is not very well established (174–179).

Once it was realized that the coordinate action of transcriptional regulation and alternative splicing (plus other forms of RNA processing) is of key importance for myogenic development (72, 75, 180), also the role of isoforms of accessory transcription factors in impaired muscle differentiation in DM attracted further attention. First evidence for their significance came from a study of members of the *MEF2* family. In vertebrates, four members of this family, *MEF2A*, *-B*, *-C,* and *-D*, are expressed. Although MEF2 members do not possess own myogenic activity, they act together with MRFs to activate and sustain the myogenic differentiation program (85, 181). As discussed earlier, MBNL1, -2, and -3 are key factors in the missplicing in DM. In their normal role, MBNL1 and -2 are positive regulators of muscle differentiation. MBNL3, on the other hand, inhibits muscle formation, by repressing adult mRNA splice isoforms (182–185). Lee et al. showed that MBNL3 influences myogenesis by disrupting *MEF2D* splicing, by favoring beta-exon exclusion (186). When the beta-including MEF2D isoform was expressed in a cell model, normal muscle differentiation was restored. Almost coincidentally, others reported on splicing changes for *MEF2A* and *-C* mRNAs. Dysregulation of *MEF2B* and *-D* and genes that are under transcriptional control of these factors, mainly those involved in calcium signaling, was found as well (88). Likewise, CELF1 upregulates translation of *MEF2A* mRNA *via* direct interaction with a GC-rich element in the transcript, causing a delay in myogenesis. Abnormal CELF1 upregulation thus explains the muscle maturation delay in DM1. For DM2 the involvement of coupled transcription-RNA processing abnormalities has not yet been documented.

First Appearance of Committed MuSCs

During early embryogenesis, a subselection of cells from the dermomyotome maintains proliferation and migrates directly to the myotome. These *PAX3*- and *PAX7*-positive cells do not express members of the MRF, homeobox or *MEF* families of transcription factors. These cells are known as the myogenic precursors that form the source of the majority of satellite cells in the adult skeletal muscle, and as such form the subject for further discussion in the next sections.

Embryonic and Prenatal Phases of Muscle Growth

Fiber Type Specification

In most vertebrates, fibers of diverse types are recognized in the embryo concomitantly with the earliest time points of muscle appearance, before innervation (187). Interestingly, slow MyHC-expressing fibers seem to form earlier than fast MyHC-expressing fibers. Hedgehog signaling is a determining mechanism required for muscle precursors to commit to the slow muscle fate. Later in development, beyond the late embryonic and fetal periods of prenatal development, the slow (type 1) fibers become less common and fast fibers (type 2) start to become the most abundant fiber type. External soluble signals, such as WNT, coming from tissues adjacent to the somites—i.e., the notochord and neural tube—plus cell–cell contacts in the embryonic niche play an important role in further growth of muscle and the specification of fiber types. Excellent reviews discuss the regulatory principles behind fiber specification (180, 187, 188).

The functional and architectural properties of fiber types that arise during embryonic and fetal muscle development are with the advancement of growth further modified by effects of physical activity, endocrine signals and muscle innervation (180, 187–189). This process continues further during postnatal life. For a better understanding of the distinct fiber type involvement in DM1 versus DM2, it is important to reiterate here that not only type 1 and 2 fate specification but also intrafusal fiber morphogenesis is under control of new combinations of transcription factors. Transcription factor EGR3, for example, is selectively expressed in sensory axon-contacted myotubes, and is a key factor for normal intrafusal fiber differentiation and spindle development (190–192). *ERB2* signaling also plays an important role (193). As was specified above, intrafusal fiber and spindle morphology are clearly affected in cDM muscles.

Similar hierarchical networks determine the fast and slow fiber specification. Involvement of transcription factors PRDM1 and SOX6 has already been well documented. PRDM1 acts as a switch that activates the slow-twitch differentiation program in cells by direct repression of fast-twitch specific genes and indirect activation of slow-twitch specific genes through limiting the activity of the SOX6 transcriptional repressor (188).

During the transition from the embryonic to the fetal phase of development, a switch occurs from basic muscle patterning (primary myogenesis) to growth and maturation of the muscle masses and the onset of innervation (secondary myogenesis). These two waves of myogenesis are mediated by distinct embryonic and fetal myoblasts, respectively, each characterized by differentially expressed genes and properties. The differentiated cells that these myoblasts produce later have also distinct features. Expression of *NFIX* is an important prerequisite for the continuation of coordination of fiber specification in the switch to fetal muscle growth. Gradual changes in the networks for transcription regulation, alternative splicing and polyadenylation thereby jointly control the differential expression of fiber type specific protein isoforms. Single muscle fiber proteomics studies have revealed hundreds of proteins that vary in level or identity between the proteomes of different fiber types. Among these are protein isoforms involved in sarcomeric architecture, contractile activity, mitochondrial and carbohydrate metabolism, calcium handling, and protein turnover (194). Differential activation of genes for fiber type specific isoforms of myosin, troponin, tropomyosin, creatine kinase, B-enolase and glycolytic, and mitochondrial enzymes is typical in this specialization (195).

Until now, not much attention was paid to differential expression of genes whose products are linked to the RNA toxicity mechanism in DM. To the best of our knowledge no publications exist on differences in expression of *MBNL1-3* or *CELF1* between fast and slow fibers. Also reports on abnormalities in expression

of *DMPK* and *CNBP* in individual fiber types in DM1 or DM2 are rare. In one early report, a decrease in *DMPK* expression in type 2a muscle fibers of DM1 patients, compared with the level in normal controls, was mentioned (196). Wheeler et al. have reported an abnormal foci count in subsynaptic nuclei and in nuclei of motor neurons at muscle-nerve junctions (67).

Muscle Progenitor Cells of Different Origin

During late fetal development the fiber composition of muscle is further defined and profound changes in the direct neighborhood of the muscle occur. Within the basal lamina formed around the muscle, the fibers are now located together with the now quiescent population of PAX3+/PAX7+ MuSCs, the satellite cells. At the end of the fetal period ~30% of myonuclei are satellite cell nuclei. The remaining ~70% are in the multinucleated fibers. Blood vessels permeate the interstitial spaces between fibers and nerve endings have established contact *via* neuromuscular junctions. During the transition to adulthood, the percentage of mononucleated cells located under the basal lamina at the muscle periphery declines sharply, due to recruitment for muscle growth and maintenance. In adult muscle, the population of satellite cells encompasses 2–5% of identifiable nuclei (11, 197, 198), which declines further during aging.

There is now compelling evidence that the skeletal muscle niches thus formed contain multiple types of cells, among them also cells with non-somitic origin, with myogenic capacity (**Figure 3**). Together with the satellite cells, the major skeletal muscle progenitor/stem cell population, these cells form the reservoir for use in skeletal muscle repair, regeneration, and maintenance (**Table 2**). Specifically, different interstitial populations of cells have now been characterized, referred to as PW1+ interstitial cells (PICs, that express *PW1/PEG3*) and β4-integrin+ cell (199). Other progenitor cells, MABs and PCs, are located in the fetal or postnatal muscle vasculature, respectively. PCs express alkaline phosphatase (*ALP*), but lack myogenic and endothelial markers (200). Using lineage tracing, it has been shown that most of these non-satellite cells are not derived from the somite, as do the true PAX3+/PAX7+ satellite cells. The vessel-derived progenitors can be traced back to Pax3+ progenitors of the paraxial mesoderm (201, 202). Both the muscle-resident satellite cells and the PCs contribute to muscle growth during prenatal and postnatal development.

We will next examine the role and fate of satellite cells in growth, renewal and regeneration of muscle. The biological significance of the other progenitor cells introduced above will be discussed further below in the context of regenerative medicine. DM pathobiology has only been studied in the satellite cell-derived myoblast population *in vitro* and by histological examinations *in vivo*. No data exist on the involvement of PICs, MABs, and PCs.

Muscle Renewal and Regenerative Myogenesis

Skeletal muscles endure a lot throughout a lifetime. First, muscle tissue has to grow in size. Then it must be constantly functionally and structurally renewed and maintained in accordance with physical demand and repaired after injury or disease. The role of the satellite cell compartment is thereby indispensable. The mechanisms by which satellite cells participate in renewal and regeneration of muscle have overt similarities to developmental myogenesis. Satellite cells follow largely the same trajectory as somite muscle cells during development, except for their start, which begins in a state of mitotic quiescence. The population of satellite cells must also be kept in check, to maintain functionality and to guarantee muscle homeostasis up to high age. This necessitates maintenance of a delicate balance between self-renewal and differentiation. In fact, evidence has accumulated showing that distinct satellite cell pools in anatomically defined muscles in the body are heterogeneous cell populations, with cells in different stages of development having different gene expression signatures (167, 199, 203–205).

Maintaining Tissue Homeostasis in Adult Muscle

In reaction to disease, injury or prolonged hypoxia, the local release of cytokines, growth factors, cell differentiation factors such as NOTCH and WNT, and other signals triggers satellite cells that are in a quiescent state. The muscle tissue itself and nearby fibroblasts and macrophages have a role in this process. The signaling starts a program of re-entry of satellite cells in cell cycle. Subsequent rounds of cell division, combined with differentiation programming, along similar lines as in embryonic development, in a subset of the satellite cells produces heterogeneity in the population. Some satellite cells retain stemness, and others become myoblasts or myocytes that undergo definite differentiation commitment (**Figure 3**).

Expansion in the muscle stem cell niche assures that some cells can remain associated with the extracellular matrix and to cells in the neighborhood. This promotes polarization and allows different cycles of asymmetric cell division, maintaining undifferentiated satellite cells, ready for reversal to quiescence (requiescence), and committed progeny for differentiation. Cells with highest expression of *NUMB*, an antagonist of *NOTCH* signaling, go back in quiescence for later self-renewal (206). Daughter cells in which p38α/β MAPK is asymmetrically activated by a so-called PAR complex, undergo commitment to myogenic differentiation (207), expand in number and form binuclear myotubes or fuse to existing fibers (168, 199, 203, 208).

A general repression of translation, mediated by the phosphorylation of translation initiation factor eIF2α, is also a key event in the maintenance of the quiescent state (209). The mitotic quiescent satellite cells express *PAX7*, *MYF5*, and *CD34* and frequently also *PAX3* (210–214). Entrance in cell cycle and progression through the myogenic lineage occurs under the control of MRFs. Activated satellite cells no longer express *CD34* and start expressing *MYOD*. Once activated satellite cells proliferate and become myoblasts, *PAX7* expression is downregulated, while *MYOD* and *MYF5* expression remain (215). *In silico* modeling of RNA processing associated with human muscle development has provided strong evidence that also the expression of *MBNL1*, *-2*, and *-3* varies during these transitions in cell state (85). In addition, *MYOD* induces the expression of p21. As mentioned earlier, p21 blocks cell cycle progression and it is involved in the switch from proliferating to differentiating myoblasts, i.e., when they become myocytes. This switch is essential for myogenic precursor cell, satellite cell, function in regenerating skeletal muscle (135, 216).

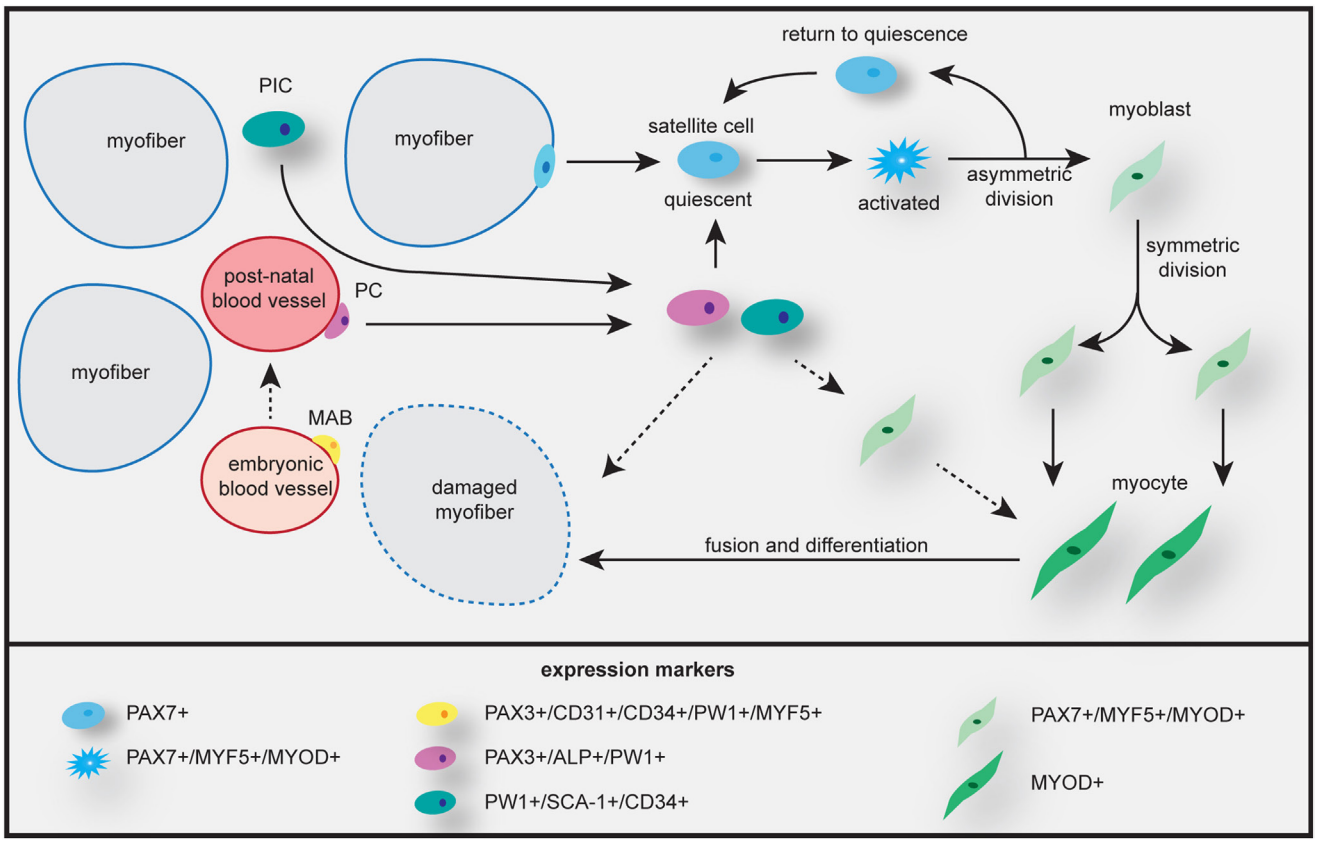

FIGURE 3 | Skeletal muscle growth, maintenance, and repair by different myogenic progenitor cells. Satellite cells from the basal lamina of the myofiber are activated and undergo asymmetric and symmetric division to generate heterogeneous progeny. Some cells undergo self-renewal and return to quiescence, others become myoblast that will proliferate and differentiate to become myocytes, which fuse to myofibers, enabling repair and/or growth. Mesoangioblasts (MABs) can contribute to muscle regeneration during embryonic growth, while pericytes (PCs) are involved in postnatal muscle growth by repopulating the quiescent stem cell population or maybe by transforming into a myoblast. Participation in growth and/or repair or direct fusion with the myofiber probably occurs along the same pathways as given for satellite cells. Uncertainties in cell fate are indicated by dashed arrows. PW1+ interstitial cells (PICs) are mostly involved in perinatal growth. Expression signatures of differentiation markers in all different cell types are listed at the bottom.

TABLE 2 | Myogenic cell types.

Cell type	Abbreviation	Definition
Muscle-resident stem cell	MuSC	Collective term for cells in (adult) skeletal muscle that can self-renew and give rise to muscle cells
Satellite cell	–	Muscle progenitor cell located in the adult stem cell niche under the basal lamina of the myofiber; upon muscle injury this cell can undergo symmetric or asymmetric cell division and produces cell progeny that undergo self-renewal or become myoblasts
Myoblast	–	General term for a mononuclear muscle progenitor cell that can proliferate or undergo terminal myogenic differentiation
Myocyte	–	Quiescent differentiated myoblast that can fuse to a myotube
Myotube	–	Multinuclear cell formed by the sequential fusion of myoblasts/myocytes, which will develop into a mature myofiber
Myofiber	–	Mature multinuclear muscle cell; the smallest contractile unit of a muscle
Induced pluripotent stem cell	iPSC	Pluripotent stem cell generated from an adult tissue cell (often a fibroblast)
Mesoangioblast	MAB	Cell isolated from the embryonic microvascular wall. A MAB has the potential to self-renew and generate multiple types of differentiated cells
Pericyte	PC	Cell isolated from the microvascular wall of postnatal tissue. A PC is capable of (trans)differentiating into other cell types when naturally or experimentally relocated to a different tissue

During normal healthy life, this whole cascade of steps for the regulation of muscle differentiation and maintenance is orchestrated by a multitude of circulating hormones, such as IGFs, FGFs, TGFs, testosterone, thyroid hormones, cytokines, and exosome-secreted signals, which are secreted locally and appear in the muscle stem cell niches. Whether and how hormonal signaling controls viability, performance and half-life of multinucleated myofibers—i.e., the bulk of muscle mass in a healthy individual—is still poorly understood, as attention of study thus far has been mainly directed toward MuSCs (208).

Failure of Tissue Homeostasis in DM Muscle

Not much is known about the fate specification of terminally differentiated multinucleated myofibers in DM. One likely possibility is that the persistent abnormalities in alternative splicing, alternative polyadenylation, and unscheduled translation of aberrant transcripts lead to the production of excessive amounts of ectopic proteins. When combined with a bulk of proteins synthesized in normal accordance with the stage of muscle during adulthood, this will create a permanent disbalance in the assembly—and perhaps turnover—of multiprotein complexes in the fiber proteome. Production of polymeric proteins by RAN translation may further create proteome abnormality. Ultimately, such imbalance will lead to a culmination of problems and to proteotoxic stress alike UPS, ER stress, or other forms of stress mentioned in this review (217). When certain thresholds are exceeded, this may lead to senescence or apoptosis. Somatic expansion of repeat length during aging may further augment the stress level, causing loss of an increasing number of fibers with disease progression and aging.

Why pathology specifically involves type 1 fibers in distal muscles of adult-onset DM1 patients and type 2 fibers in proximal muscles in DM2 needs more study. The answers may not be found only in the mature fibers themselves. They also must be sought in differences between DM1 and DM2 in the fitness of their satellite cell pools, or in the modes of recruitment of satellite cells for the regeneration of damaged fibers. As addressed before, the relevance of the satellite cell pool becomes early apparent in cDM patients, who are born with an excessive number of satellite cells and have thin muscle fibers, typical markers for immature muscle fiber growth, diminished recruitment, and delayed differentiation (125, 126). Severe disruption of RNA processing is the key element in the diminished capacity of muscle precursor cells in muscle formation in cDM, as recently demonstrated by combining transcriptome profiling of muscle tissue from patients and mouse models (85). In adult-onset DM1, the number of satellite cells are increased in distal but not in proximal muscles (218). Late myogenic differentiation markers are not fully expressed (219). In cell culture, DM1 and DM2 myoblasts show a premature proliferative growth arrest compared with healthy myoblasts (5). Combined, these observations point to a situation in which the regenerative capacity of satellite cells induced in response to fiber dystrophy is constitutively impaired (36, 220).

To understand muscle wasting in greater detail, we first need to know whether the cellular effects of fiber dystrophy are indeed dominant over those of regeneration failure. Then, to deconvolute the complexity of DM further, molecular analyses are needed. First, we need to know whether failure in pools of satellite cells to adequately balance asymmetric and symmetric division and/or subsequent loss of regenerative potency after myogenic commitment could be involved. Underlying mechanisms and differences between DM1 and DM2 muscles therein must be analyzed. Other studies should be concentrated on the loss of functionality, stability, and viability of fibers in DM1 and DM2. Preferably cell- and lineage-tracing studies should be non-invasive and concentrated on the fate of individual myoblasts, myocytes and muscle fibers over longer periods of aging. For obvious reasons, these types of longitudinal analyses of individual cells are virtually impossible for human muscle. However, tracing of cells during development and maintenance in muscles of animal models of DM will also become challenging.

Premature Muscle Aging in DM

From a clinical perspective, various symptoms of DM1 can be seen as a manifestation of progeria or accelerated aging (221–223), while aging-like symptoms are not as apparent in DM2. The progression of dystrophy in skeletal muscle in DM1 patients shows similarities with sarcopenia, i.e., age-related loss of muscle mass, strength, and function (5). Experimental evidence is mostly indirect and based on descriptive studies, wherein histopathological features such as grouped atrophy, fiber size variability, and central nuclei were investigated in sarcopenic and DM1 muscle (224). Also compelling ultrastructural and molecular evidence was provided, showing that alterations in RNA metabolism in myonuclei from DM1 patients and in aging muscle share similarity (221, 222, 225).

The mechanisms underlying age-related muscle wasting and weakness are probably diverse and not well understood (226). A recent single-fiber proteomics approach showed that the senescence of type 1 and 2 muscle fibers during aging in healthy donors is characterized by several diverging mechanisms. Differential adaptations in cellular carbohydrate and energy metabolism and the networks for protein quality control and proteostasis were among the most conspicuous changes in slow and fast fibers (194). Earlier profiling studies had pointed to a glycolytic to oxidative shift (227) or non-specified overall changes caused by aging in whole human muscles (228). The numerical loss and the loss of functionality of MuSCs, rather than fibers, with aging have attracted until now more attention, as they provide an explanation for the regenerative failure of aged muscle. For more details on the molecular and cellular findings, we refer the reader to comprehensive reviews on this topic (229–231).

Within the networks for muscle regeneration and maintenance during aging, only a few players and processes have been identified that bear direct relevance for DM1 and DM2 pathophysiology. DNA repair is one important issue. Nuclei in resting satellite cells and in muscle fibers are highly efficient in DNA repair through non-homologous end joining, explaining why repeat expansion predominantly occurs in these cells (232). Ongoing somatic expansion of the (CTG•CAG)n and (CCTG•CAGG)n repeats due to DNA repair in quiescent cells may thus be an important factor in impaired muscle regeneration in patients (23). Whether age-induced changes in the production of mitochondrial reactive oxygen species also have an effect must still be analyzed. Accumulation of reactive oxygen species damage is a known contributing factor to repeat expansion (233, 234). Age-dependent changes in oxidative metabolism must, however, have different effects in DM1 and DM2 muscles, as the affected fiber types differ in both forms of disease.

The shortening of telomeres is probably not a major contributor to muscle aging, although effects on premature senescence of DM2 satellite cells have been suggested (220). The situation in DM1 is less clear. Satellite cells in cDM patients did have a higher telomere shortening rate, but they entered senescence before reaching a critical length. This argues against a determining role

of telomere shortening as an explanation for diminished differentiation capacity in cDM muscle (218, 235).

A more likely candidate mechanism for the premature growth arrest in DM1 muscle precursor cells is activation of the *p16^{Ink4a}*-pathway that leads to CDK4 inhibition and cell cycle arrest. p16 accumulates in myoblasts from DM1 patients in response to (CTG)n-related stress (220, 235), resulting in impaired regeneration and atrophy. As mentioned, aging-like symptoms are not so apparent in DM2 patients and the *p16* pathway appears not to be altered in DM2 satellite cells and fibers (220, 221). Finally, increased p38/MAPK signaling is a typical feature of aged satellite cells (236), but evidence for p38 signaling abnormalities in DM muscle is lacking. Also evidence for the involvement of apoptosis in DM muscle wasting is still limited (159, 163, 237).

An interesting test for the question how DM effects are superimposed on senescence of normal aging would be to study the effects of ablation of *p16^{Ink4a}*-expressing cells in muscle of DM mouse models. This is possible with use of a genetic approach recently developed by Baker and co-workers in Van Deursen's laboratory (238) and also with drug treatment (239). Any alteration in muscle health in the DM mice would provide us with novel insight in the causative effects of expanded repeats on the viability of progenitor cells in muscle.

Stress Signaling in Adaptation to Regenerative Failure, Effects of Disease, and Aging

Adaptation to cell-autonomous stress in muscle depends on a combination of intrinsic and extrinsic signaling mechanisms. Many intracellular pathways are known that protect cells against stress from for example DNA damage, proteotoxicity, and calcium-mediated excitotoxicity (240). Best-known are the P53, AKT, and NRF2 pathways, but these pathways have not yet been intensely studied in skeletal muscle of DM patients.

Changes in intercellular communication may also fulfill a central role. Many of the secreted hormones and factors that are exchanged between cells and orchestrate myogenesis and regeneration have been extensively discussed in some of the reviews mentioned above (208). Among these are the WNT proteins, HGF, FGFs, IGF-1 splice variants, myostatin, and TGF-β (241). Although the working mode of these secreted factors is reasonably well understood, it is not always clear what cell types in the muscle stem cell niche are in the secretory and/or the responding mode. Satellite cells from cDM patients secrete increased levels of prostaglandin E2 *in vitro*. This secretion is controlled *via* upregulation of cyclo-oxygenase 2, mPGES-1, and prostaglandin E2/EP4 receptors. A direct consequence of the prostaglandin E2 upregulation is a decrease in intracellular Ca^{2+} and impairment of fusogenic capacity of the satellite cells (242). It was also shown that cDM muscle and primary myoblast derived thereof produce a higher level of IL-6, indicative for increased activity of this myokine signaling pathway (52).

Another conspicuous observation was that variation in the level of CELF1, as seen in cDM muscles, causes imbalance in the production of subunits for the signal recognition particle in the ER-secretory pathway (243). CELF1 misregulation may thus be coupled to changes in the secretory route for extracellular matrix proteins. Others confirmed that production of ECM proteins is indeed altered in muscle of a mouse model for DM1 (244). Taken together, this is compelling evidence that the hormonal and ECM environment of progenitor cells in the DM muscle are changed. There is no doubt that this will compromise the "cry-for-help" communication in DM muscle and its adaptive regenerative capacity in response to accelerated fiber decay due to repeat stress.

MicroRNAs (miRNAs) and Other Non-Coding RNAs in Muscle Homeostasis

MicroRNAs have a critical role in cellular stress responses, differentiation, proliferation, and apoptosis in muscle (245, 246). MiRNAs are short, highly conserved non-coding RNAs that occur in all cell types, where they regulate the stability and the translational efficiency of target mRNAs (247). Multiple miRNAs that regulate differentiation and stress adaptation of skeletal muscle, referred to as myomiRs, exist (248). Among them are miR-1, -133a, -133b, -206 (the most abundant miRNA in skeletal muscle), and miR-208. Expression of these miRNAs is regulated by transcriptional networks involving *MEF2*, *MYOD*, *SRF*, and *TWIST1* (249, 250). Non-muscle specific miRNAs that regulate differentiation and regeneration after muscle injury are miR-181, -221, and -222 (251).

Myoblasts and myofibers utilize exosome-clustered extracellular miRNAs as paracrine and endocrine communication signals to regulate homeostasis and regeneration. Extracellular myomiRs are elevated during perinatal muscle development and after exercise-induced muscle regeneration. Also in primary human myoblast and C2C12 cultures, these extracellular myomiRs were elevated and appeared to be released selectively as a consequence of the differentiation process (252).

Myotonic dystrophy type 1 and 2 profiling studies showed that deregulation of intracellular miRNA content in muscle, and extracellular extrusion *via* exosome secretion is a hallmark of disease. Eight miRNAs were found to be significantly deregulated in the serum of DM1 patients (i.e., miR-1, -27b, -133a, -133b, -140-3p, -206, -454, and -574) (253). Earlier work had shown upregulation of miR-1, -206, and -335 and downregulation of miR-29b, -29c, and -33 in DM1 biopsies compared with controls (254, 255). Moreover, cellular distribution of miR-1, -133b, and -206 was altered in DM1 skeletal muscles. Koutsoulidou et al. demonstrated that appearance of miR-1, -133a, -133b, and -206 in serum correlated with the progression of muscle wasting in DM1 patients. All four miRNAs were found encapsulated within exosomes in the circulation (256). Cell and animal model studies suggest that *MBNL* expression is controlled by miR-277 and -304 (257), and miR-30-5p (258), and that this regulatory network could be involved in inhibition of myogenic differentiation in DM1. In DM2 muscle biopsies, the levels of 11 miRNAs were found to be significantly modulated (259). Of these, three also showed modulation in DM1 patients (i.e., miR-193bp, -208a, and -381). Expression levels of the other eight (i.e., miR-34a-5p, -34b-3p, -34c-5p, -125b-5p, -146b-5p, -193a-3p, -221-3p, and -378a-3p) fitted in a unique DM2 profile. The differences in miRNA expression profiles might contribute to the differences in muscle pathobiology between DM1 and DM2 (259).

Long non-coding RNAs and circular RNAs (circRNAs) may also have a role as regulators of muscle homeostasis and gene expression (260, 261). LncRNAs are arbitrarily defined as RNAs >200 nts without overt protein-coding potential, of which at least 5,000 have been identified so far (262). CircRNAs are shaped as covalently closed molecules that lack 5′ and 3′ ends. They are expressed by a high number of genes and are highly conserved among species (263). Although little is known about the function of these RNA species, it has been shown that they can modulate gene expression by competing for miRNA or protein binding, or with regular mRNA production (264–267). Some lncRNAs are important players in muscle differentiation (268, 269) and involved in the pathomechanisms for Duchenne muscular dystrophy (270) and facioscapulohumeral muscular dystrophy (271). *Malat1*, one of the most abundant lncRNAs, was recently found to slow down myogenic differentiation in mice by interference with MyoD-binding loci and formation of a repressive histone-methylation complex. After the onset of differentiation, miR-181 targets *Malat1* RNA for breakdown to release the repression (272). Our group has published evidence that *DM1-AS* transcripts belong to the class of lncRNAs. After alternative splicing and alternative polyadenylation, different (CAG)n repeat containing *DM1-AS* RNA isoforms are produced. Like many other lncRNAs, *DM1-AS* RNA is expressed at very low copy numbers per cell, in parallel with (CUG)n-containing *DMPK* mRNA (273). It remains to be seen whether expanded *DM1-AS* transcripts have an effect on DM1 myopathy, either in isolation or together with expanded *DMPK* transcripts. No circRNAs that are possibly linked to DM have so far been identified, but considering the fast developments, we might soon hear more from this field of research.

REGENERATIVE MEDICINE FOR DM: PROGENITOR CELLS AS SOURCE FOR MUSCLE HEALING

Use of MuSCs

The satellite cells, the adult MuSCs located between the basal lamina and the sarcolemma of the multinucleated myofibers, form the main pool of progenitors for skeletal muscle regeneration *in vivo* (**Table 2**). A large body of research has been devoted to the isolation, propagation, and genome tailoring of these cells, as they are the most logical candidates for use in future cell-based therapies, capable of restoring tissue homeostasis, and enhancing muscle repair in patients with myopathies.

Identification and Isolation of MuSCs

For an *ex vivo* approach to gene therapy of DM in coupling with muscle cell transplantation the availability of sufficient quantities of MuSCs is a prerequisite. Use of these cells for regenerative medicine in DM, whereby different groups of skeletal muscles are differentially affected, will not be simple. Indeed, although all satellite cells should be considered remnants of embryonic development prepared to recapitulate muscle development in the event of muscle damage (197), it is only a fraction of this heterogeneous population that fully preserves the self-renewal potential and myogenic capacity, when brought in *in vitro* culture. This seemingly stochastic nature of fate adoption, which is associated with a high-degree of heterogeneity and plasticity of the satellite cell population in the natural environment of the muscle (203), is a complicating factor during the period that they regain proliferative activity as myoblasts.

Another complicating factor is that MuSCs have the same embryonic origin as the muscle in which they reside. Most skeletal muscles of the trunk and limb are derived from somites, but head muscles originate from cranial mesoderm. These distinct origins specify distinct genetic programs (274), which may be permanently associated with the intrinsic properties of MuSCs (275). More study is thus needed to verify whether the distinct origin is also a determining and retained factor for capacity to participate in regeneration of muscles in different locations of the body, or whether differences are smoothened out upon maintenance of cells in *in vitro* culture. Lastly, aging of the donor seems to render the MuSC pool increasingly dysfunctional, as MuSCs progressively lose their potency due to cell death and terminal differentiation. Hence, aging forms an extra problem in cases where the patient's own progenitor cells must be used for cell therapy to circumvent immunological problems, and especially so in patients with late-onset genetic myopathies like in DM2 or certain cases of DM1.

Skuk and colleagues came up with three properties that cells used for repair of damaged and replacement of lost muscle fibers should have: (i) ability to fuse with pre-existing myofibers, (ii) ability to form new myofibers, and (iii) ability to produce myogenically committed stem cells (276). This means that the MuSC's capacity to participate in all aspects of muscle homeostasis must be maintained during expansion *ex vivo*. Novel strategies for satellite cell culture and preservation of self-renewal capacity before transplantation into muscle have now become available. Cell culture on pliable soft hydrogel matrices, in combination with pharmacological inhibition of p38/MAPK signaling (277) or culture on natural biopolymeric films (278) simulate the conditions of the muscle stem cell niche and help to preserve MuSC quiescence and enhance their self-renewal capacity. Also modulation of *PAX7* expression may thereby be of help (279).

Transplantation of MuSCs: Preclinical Studies Only

Currently, the use of MuSCs in cell-based therapies is almost impossible. As demonstrated in animal model studies, MuSCs cannot be delivered systematically to all muscles in the body (280). Upon intravenous delivery they accumulate in the lung, liver, spleen, and kidney but not in skeletal muscle. One of the largest technical hurdles that limit the feasibility of MuSC transplantation is, therefore, associated with the route of administration, i.e., intramuscular injection (**Figure 4**). Initial trials aiming to regenerate skeletal muscle by local injection of donor myoblasts failed due to their poor survival and limited ability to migrate more than a few millimeters away from the site of injection (281–283). Upon engraftment, these satellite-derived myoblasts could not efficiently repopulate the satellite cell niche, and therefore were not able to contribute significantly to muscle regeneration (284, 285). Future work is necessary to find out whether some of these issues might be overcome by increasing the numbers of engrafted

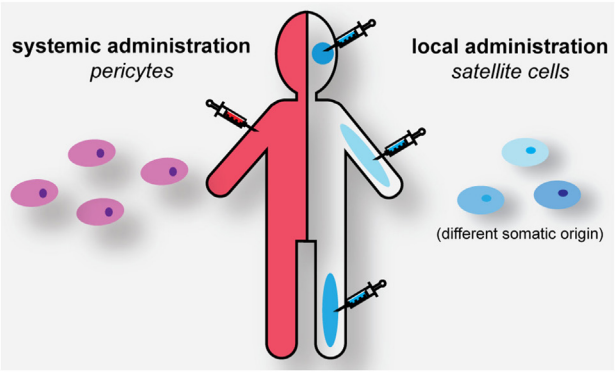

FIGURE 4 | Strategies for cell-based muscle therapy in myotonic dystrophy. Genome-edited autologous or HLA-matched pericytes (PCs) can be administered systemically for muscle healing. Genome-edited or HLA-matched satellite cells need to be engrafted locally in the corresponding muscles to have a regenerative effect.

cells, or by better preservation of their stemness during *in vitro* propagation as discussed above.

Further work on MuSCs in culture is, therefore, necessary. For application in basal and translational research in DM, immortalized myoblast cell lines are available. These lines have preserved the molecular hallmarks of disease, including splicing abnormalities and repeat RNA-MBNL foci (86) and were generated by lentiviral-mediated expression of the catalytic subunit of the human telomerase (*TERT*) and *CDK4*, the natural p16 ligand. Immortalized cells constitute an unlimited source of cells for evaluation of compounds with therapeutic potential. Immortalization *per se* may not be detrimental for the ability of muscle progenitor cells to serve in therapeutic engraftment experiments in mice, as already shown earlier for these type of cells and for SV40-TAg^{ts} immortalized cells (286, 287). However, for obvious reasons use of these transformed cells for human studies will probably remain restricted for *in vitro* work.

Use of Stem Cells From Non-Muscle Origin

The continuous search for stem cells with potency for transdifferentiation and adaptation from other sites than within the muscle basal lamina (288–295) has led to the identification of entirely unexpected cell types with muscle progenitor capacity. Among these are the vessel-associated MABs and PCs the best-known examples (**Figure 3**) (199).

Identification and Isolation of PCs and MABs

The participation of MABs and PCs in myogenic differentiation and regeneration *in vivo* is still a poorly understood phenomenon. There is, however, compelling evidence that these cell types have great potential for boosting muscle repair in regenerative medicine. One advantage, which MABs and PCs may have, is that they rapidly acquire unlimited lifespan and maintenance of multipotency, making them ideally suitable for the generation of replenishable pools of transplantable cells. Skeletal muscle tissue itself is the most effective source for PCs with this potential (200, 296). Their isolation can be accomplished by using explant culture methodology (297, 298), eventually in combination with enzymatic dissociation and FACS for surface markers (200, 299). PCs with skeletal myogenic potential can be distinguished by expression of *ALP* (200, 300) and new biomarkers for therapeutic potency, like *PW1/Peg3*, a regulator of myogenic ability and migration capacity in PCs, MABs and satellite cells, have recently been identified (301). Expression of *PW1/Peg3* is high in both MABs and PCs and its level of expression correlates with their progenitor cell competence. Moreover, lack of *PW1/Peg3* expression abrogates the cells ability to cross the vessel wall and to engraft into damaged myofibers through the modulation of the junctional adhesion molecule. PCs and MABs are expandable *in vitro* as a relatively homogeneous population and transducible with viral vectors for genomic editing.

Engraftment of PCs and MABs

Pericytes and MABs are able to systemically reach the target tissue, where they engraft and differentiate toward the myogenic lineage (**Figure 4**). One possible complication, however, is that adequate measures are necessary to ensure that myogenic commitment of these vessel-derived progenitor cells is appropriately stimulated, while adipose and fibrogenic commitment must be avoided. Several recent publications have implicated a role for a PC subtype in fibro-adipose infiltration of tissues (299, 302). Consistent with age-dependent changes in regeneration capacity seen before, this property seems to be more present in PCs isolated from aged individuals. PCs failed to differentiate or participate in myofiber repair following injury, but contributed to enhanced fibrous tissue deposition within the interstitial space in aged muscle (299, 303–305). Further work is thus necessary to see whether PCs and MABs are truly the ideal candidates for use in regenerative medicine in DM patients.

Translational studies in the GRMD dog model of myopathy demonstrated that *ex vivo* cultivated PCs can indeed adopt myogenic fate when exposed to injury factors *in vivo* and are able to directly differentiate into skeletal muscle or replenish the SC pool *via* activation of *Pax7*, *Myf5*, or *MyoD* at the onset of differentiation (200). For the GRMD model "a remarkable clinical amelioration and preservation of active motility" was seen (306). The first human clinical study with PCs was published in 2015, investigating primarily the safety of intra-arterial transplantation of *HLA*-matched donor cells. This exploratory clinical trial was performed in five Duchenne patients, in combination with immunosuppressive therapy. Clinical laboratory and MRI analysis revealed that the study was relatively safe. Unfortunately, the effects of the cellular therapy on muscle function were inconclusive.

Although the possibility for systemic administration is one of the strongest arguments for preference of vessel-associated progenitor cells over satellite cells, there is also concern, as blood flow in the artery of microvasculature downstream of the injection site might get disrupted (307). Moreover, a fraction of the injected cells might become trapped in filter organs decreasing the amount of cells available for engraftment into dystrophic muscle (200). Modification to improve homing to damaged muscles (308) or altering cell surface (309) needs to be studied in more detail to

address these possible problems. For DM, research regarding the potential and use of MABs or PCs for therapy is entirely missing.

Induced Pluripotent Stem Cells (iPSCs)

Also whole new approaches toward deriving myogenic progenitor cells from pluripotent embryonic stem cells and iPSCs are now being developed (310–315). Generation of iPSCs from fibroblasts of DM1 and DM2 patients has been published (316–321). Recently, a revolutionary new method to direct human iPSCs to adopt muscle progenitor cell identity and create a renewable source of muscle progenitors for regenerative medicine was developed. Hicks et al. found that the use of FACS of cells for two cell surface markers, *ERBB3* and *NGFR*, and treatment with a TGFβ inhibitor gave an enormous enrichment for progenitors with regenerative potential during engraftment (322). Further work is necessary to verify whether simple extrapolation of these animal model transplantation findings to the human situation is possible.

Cell-Based Therapy in Combination With Genome Editing

To prevent immunological problems linked to MuSC transplantation, the use of progenitor cells from HLA-matched donors or autologous cells from patients is strongly advisable. For DM1 and DM2 cells, this implicates that genome editing must be employed to normalize the length of the expanded repeats or the synthesis of the toxic RNAs must be otherwise permanently prevented. With the advent of gene editing tools such as ZFN, TALEN, and CRISPR/Cas9 this now has become a realistic goal. Specifically for DM1, a small number of gene editing studies have been published recently, all aiming at the prevention of the presence of toxic, expanded repeat-containing RNA.

Gao et al. inserted a poly(A) signal upstream of the expanded (CTG)n repeat in *DMPK* in iPSCs. This insertion led to premature termination of transcription and prevented production of (CUG)n repeat containing transcripts. As the *DMPK* mRNAs were now missing the repeat-containing 3′ end, a healthy stem-cell pool was created (320). Cardiomyocytes derived from these iPSCs reverted to normal splicing for a number of pre-mRNAs known to be misspliced in DM1.

Pinto et al. used a deactivated Cas9 variant to impede synthesis of expanded (CUG)n RNA during transcription (323), while Batra et al. (324) used an RNA-targeting Cas9 to eliminate toxic expanded RNA after production. Both studies showed efficient elimination of cellular hallmarks of disease, but the strategies used seem not well suited for permanent transformation of muscle progenitor cells and prevention of repeat RNA effects.

More permanent effects for use in cellular strategies may be expected from removal or trimming of the (CTG•CAG)n repeat expansion in the DM1 locus, creating permanently normalized *DMPK*/*DM1-AS* alleles. Our own group and others have published that excision of the repeat (and short flanking sequences) can be achieved by dual CRISPR/Cas9 cleavage at either side of the repeat (113, 325). Repeat removal had no adverse biological effects on DMPK isoform production and normalized splicing and myogenic capacity. Notably, CRISPR/Cas9 cleavage in the vicinity of the repeat was associated with a risk of uncontrollable DNA rearrangements across the area (113, 325). Also off-target alteration elsewhere in the genome is a known danger in the application of CRISPR/Cas9 technology. Hence, careful characterization and selection of cell clones with only the desired genome alterations should become routine steps in future cell-based therapeutic strategies.

Use of repeat-corrected cell therapy may serve to halt the degenerative process, or delay or prevent the onset of disease when applied upon first diagnosis with DM. In parallel, more work will be devoted to the development of modalities for direct *in vivo* treatment of DM, with vector-mediated gene-editing therapy. Finding ways for improvement of the quality of life of patients with DM will remain the goal of a large variety of future translational studies.

AUTHOR CONTRIBUTIONS

LA and CA designed the figures. LA, CA, and BW drafted the contents of this review, and together with DW wrote the text. All authors contributed equally to critical reading of the final manuscript, including text and figures.

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Cells of Matter—*In Vitro* Models for Myotonic Dystrophy

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Myotonic dystrophy type 1 (DM1 also known as Steinert disease) is a multisystemic disorder mainly characterized by myotonia, progressive muscle weakness and wasting, cognitive impairments, and cardiac defects. This autosomal dominant disease is caused by the expression of nuclear retained RNAs containing pathologic expanded CUG repeats that alter the function of RNA-binding proteins in a tissue-specific manner, leading ultimately to neuromuscular dysfunction and clinical symptoms. Although considerable knowledge has been gathered on myotonic dystrophy since its first description, the development of novel relevant disease models remains of high importance to investigate pathophysiologic mechanisms and to assess new therapeutic approaches. In addition to animal models, *in vitro* cell cultures provide a unique resource for both fundamental and translational research. This review discusses how cellular models broke ground to decipher molecular basis of DM1 and describes currently available cell models, ranging from exogenous expression of the CTG tracts to variable patients' derived cells.

Keywords: dm1, cells, cultured, CTG repeats, models, biological, pathophysiology, human cells

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INTRODUCTION

Myotonic dystrophies (DM) are a group of dominant disorders that are among the most prevalent neuromuscular diseases in adults (1). The main characteristics of these multisystemic diseases are myotonia, progressive muscle weakness and wasting, cardiac-conduction defects, cognitive impairments together with other endocrine dysfunctions (1). Two DM forms have been identified so far: type 1 (DM1 also called Steinert Disease) and type 2 (DM2, previously known as PROMM), which is generally less severe than DM1. Both share a similar molecular mechanism in which deleterious expansion of microsatellite repeats in non-coding regions, (CTG)n in 3′UTR of dystrophia myotonica protein kinase (*DMPK*) gene in DM1 (2–4) and (CCTG)n in intron 1 of *CNBP* gene in DM2 (5) are transcribed into expanded C/CUG-RNA that are retained in the nucleus as discrete foci. These ribonuclear foci sequester muscleblind-like (MBNL) RNA-binding proteins, resulting in their functional loss and consequently, RNA metabolism alterations (6–11). Thus, misregulation of alternative splicing events within downstream effector genes were found in striated muscles of DM1 patients and associated with clinical symptoms, such as insulin resistance, myotonia, muscle weakness, and cardiac defects (12–18).

During the past 20 years, several animal models, including mouse, fly, zebrafish, and worm have been developed to investigate DM1 pathophysiologic mechanisms. They largely contributed to the current state of the art on myotonic dystrophies, which also benefited from research performed on cell cultures. At present, more than 100 years from the first descriptions of Steinert disease, there is still a need for cellular models to decipher disease-related molecular mechanisms and evaluate therapeutic approaches before *in vivo* validation. Because DM is a multisystemic disease affecting many tissues and cell types, various cell models are required to cover all DM-associated defects. Thanks to

technological progresses, we have access to new cellular models allowing more comprehensive and adequate studies. Herein, we will discuss the use of *in vitro* cell models through the advances in myotonic dystrophy research and describe the available cellular models, from exogenous expression of CTG repeats to patient's derived cells, which were developed for the study of DM1.

CELLULAR MODELS IN DM1 RESEARCH HISTORY

In the early 1900s, Dr. Hans Steinert provided for the first time a detailed description of a neuromuscular disorder characterized by dystrophic progression with myotonia and degeneration of skeletal muscle (1). Since then, Steinert's disease that was renamed as myotonic dystrophy type 1 or DM1 by the International Myotonic Dystrophy Consortium has been extensively investigated at both clinical and pathophysiologic level. Even before the discovery of the mutation responsible for DM1, primary cells derived from DM1 patients have been used to uncover differences in behavior or cytochemistry (19–22) to study metabolism (23–26) or to understand mechanisms leading to symptoms described in patients, like widely observed insulin resistance (27–30). However, besides learning about the clinical, physiological, and cellular manifestations of DM1, it was essential to define the molecular bases of the disease. The first breakthrough came in 1992, when the mutation responsible for DM1 was identified as an unstable CTG expansion within the 3′ non-coding region of the *DMPK* gene (2–4, 27, 31, 32). The next challenge was to understand how this expansion leads to molecular and cellular defects observed in DM1 cells. As it became striking that mutant *DMPK* mRNA was altered in DM1, the use of different cellular models provided initially confusing conclusions about its expression in the disease (33, 34). Nevertheless, the observation that the level of mutant *DMPK* mRNA decreased when the size of the repeats increased, led to the hypothesis that expanded repeats were rather impairing post-transcriptional processing of the mutant DM1 allele (35). The compelling evidence for this postulate came shortly after, when discrete ribonuclear foci were reported for the first time in DM1 fibroblasts (36). Additionally, experiments performed with patient-derived myoblasts and fibroblasts determined that mutant *DMPK* transcripts, while correctly spliced and polyadenylated, were not exported to the cytoplasm but retained in the nucleus (37, 38), causing approximately 50% reduction of the DMPK protein levels in DM1 myoblasts (39). These findings obtained from patient-derived cells gave rise to the idea of a RNA gain-of-function mechanism in DM1. This concept was proposed following the identification of a RNA-binding protein, CELF1 (also called CUG-BP) that could bind to single-stranded UG motifs and is aberrantly accumulated in the nucleus of cells derived from DM1 patients (40–43). Upregulation of CELF1 and its splicing regulatory activity have been associated with abnormal splicing of downstream targets, suggesting a trans-dominant effect of CUG repeats on RNA processing in DM1 (18, 44) which was further confirmed in cell models overexpressing exogenous CUG expanded tracts with increasing lengths (16). Finally, at the beginning of the 2000s, a second breakthrough has been reached with the identification of RNA-binding proteins that bind specifically to CUG repeats proportionally to the size of the expansion (45). These proteins belong to the MBNL family, which includes three paralogs (MBNL1, MBNL2, and MBNL3), and all of them are sequestered within the nuclear RNA foci in DM1 patient cells (46). Among their functions, MBNL proteins are splicing regulatory factors that control developmental switch between fetal and adult isoforms of many transcripts (47). Thus, titration of MBNL proteins by nuclear CUGexp-RNA results in alternative splicing misregulations of several pre-mRNAs in DM1, and some of them are associated to DM1 phenotypic features, establishing the deficiency of functional MBNLs as a central cause of the disease (12–18, 48–51).

CELL MODELS EXPRESSING EXOGENOUS CTG REPEATS

Several years after the identification of the mutation, the expression of exogenously expressed CTG tracts in cellular models was widely used as a tool to confirm the direct role of the repeats in the pathologic mechanisms of DM1. The repeats, usually inserted in the 3′UTR of a truncated *DMPK* gene commonly under the control of a CMV promoter, are transiently or stably expressed in well-characterized human or murine cell lines, such as HeLa, HEK, or C2 cells. Even if they are lacking the entire genomic context of the CTG expansion and its own specific promoter regulation, they still recapitulate several DM1-associated features like the formation of ribonuclear foci that colocalizes with MBNL proteins and the splicing defect (16, 52, 53). Thus, they provide fast and reproducible tools for informative screening readout. Up to date, several constructs containing expanded CTG repeats have been described. A construct expressing interrupted 960 CTG repeats has been used in a wide range of studies including molecular mechanism investigations and validation of therapeutic approaches (54). To ensure the stability of the expansion, the CTG tracts are interrupted with TCGA sequences every 20 repeats (16). Several similar constructs with different lengths of CTG repeats and/or promoters were developed (16, 52, 55, 56), however, the potential impact of these interruptions is not well defined yet. Additionally, constructs expressing short but pure repeats were also developed. Stable muscle cell lines expressing 200 CTG showed nuclear aggregates of mutated RNA that may cause disruptions in myogenic differentiation according to the 3′UTR-*DMPK* environments of the CTG repeats (57, 58). Overexpression of large pure repeats is more challenging due to their instability and technical issues associated with the cloning of long tracts of CTG repeats that substantially restricts their length (59, 60). However, some works partially overcame this constraint by expressing plasmids reaching 800 and 914 uninterrupted CTG repeats in the 3′UTR context of the *DMPK* gene or in inducible construct expressing GFP, respectively (10, 61–63).

In vitro cell models expressing exogenous CTG repeats have been widely used for small molecules screenings, therapeutic approaches, or molecular investigations (16, 54–56). However, these models may encounter some limitations associated with the level of CTG overexpression that is not under the control of the

endogenous *DMPK* promoter and the absence of the complete *DMPK* genomic context that could limit their use in specific tissue or molecular mechanism studies.

DM1 PATIENTS' DERIVED CELL MODELS

Cells obtained directly from patients are of great utility in modeling human genetic disorders if they reproduce molecular hallmarks of the disease. Regarding DM1 patients' derived cells, they express the whole range of mutation lengths observed in affected individuals within their natural genomic context and reproduce other canonical features of the disease (**Figure 1**) such as CUGexp-RNA foci that colocalize with the MBNL family members (45, 46, 64–70), alternative splicing misregulations (48, 65, 67, 70–73), and alterations of metabolic pathways (74, 75). However, considering variable parameters, like for instance samples from patients with different forms of the disease (from congenital to adult), culture conditions, or replicative senescence of primary cells, one should be aware of experimental variability between them. The generation of patient's cell lines or stem cells with their vast reprogramming abilities represent additional tools for deciphering molecular DM1 pathogenesis but also for translational research including drugs screening (72, 76) and therapeutics development (65, 70, 77–79).

Human Primary Cells

Currently in the DM1 field, primary cell cultures mainly consist of dermal fibroblasts and skeletal muscle cells, also called myoblasts. Isolated directly from patient's tissue by either an enzymatic digestion of the extracellular matrix or an explant technique (small sliced pieces of biopsy from which cells grow out), primary cells potentially maintain physiological characteristics of their origin tissue environment. Primary fibroblasts are often used because of their relative accessibility from patients and their ease of manipulation in culture. On the other hand, primary myoblasts have the advantage to initiate a myogenic differentiation that results in their fusion into multinucleated cells or myotubes (**Figure 1**). Concomitantly, the expression of the *DMPK* gene is upregulated during the myogenic differentiation process (39) and differentiated DM1 muscle cells conserve some features found in DM1 muscles such as alternative splicing misregulation of muscle-specific transcripts (49, 78–88). Given the relative difficulty to have access to muscle biopsies of dystrophic patients, an alternative source of muscle-like cells was designed. Thus, primary fibroblasts were transduced with a viral construct expressing the key myogenic factor MYOD1 to force the expression of the myogenic program. This trans-differentiation leads to the formation of differentiated muscle-like cells expressing muscle-specific transcripts presenting similar splicing abnormalities that those found in DM1 primary myoblasts and patients' muscles (18, 89, 90).

However, working with primary cells has also some constraints. Asides from the limited accessibility and availability of biopsies from patients, all somatic cells enter into replicative senescence after a define number of divisions that is inversely correlated with the age of the donor (91, 92). This phenomenon is even more pronounced in DM cells as their proliferative capacity is reduced when compared with age-matched control due to a premature entry into replicative senescence (81). Another difficulty, except the lengths of the repeats itself, is that primary cells could reflect the variability of the individual they are isolated from. Indeed, the age of the donor (fetal vs. adult), the tissue origin (distal vs. proximal muscle), and impairment, and the severity of the patient symptoms could influence cells behavior when grown in culture. In addition, various optimizations of cell cultures and medias or manipulation conditions may potentially lead to discrepancies between results.

Immortalized Human Fibroblast and Myoblasts

To circumvent the limitation of replicative senescence and keep the cells in a proliferative state, immortalized cell lines from

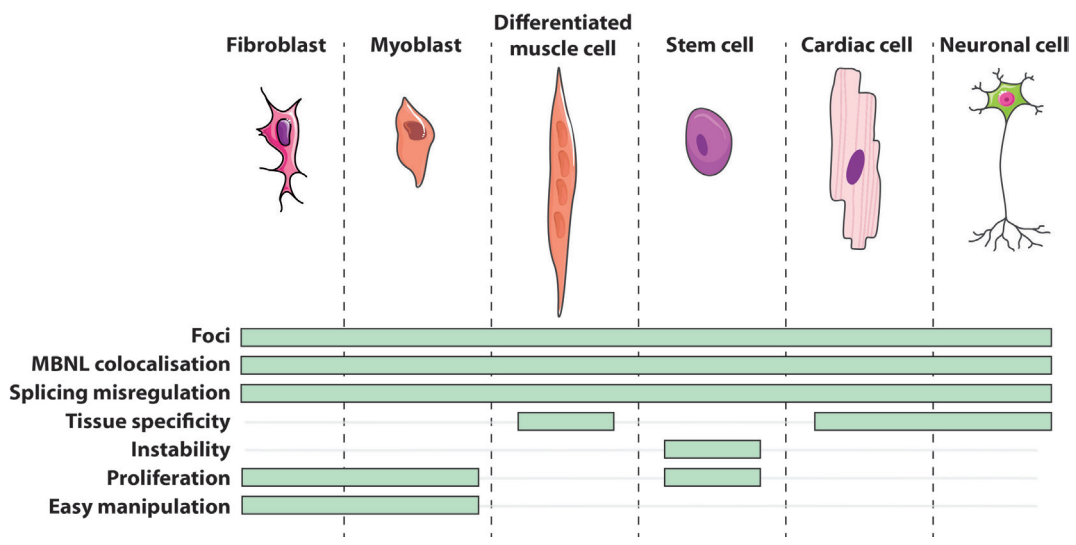

FIGURE 1 | Features of DM1 cells *in vitro*.

DM1 primary fibroblasts, trans-differentiated fibroblasts, and myoblasts have been established (**Figure 2**) (71, 76, 93–95). The immortalization process of human fibroblasts requires the stable re-expression of the human telomerase (hTERT) to prevent the excessive shortening of telomeres that triggers the entry in replicative senescence. Additional inhibition of the dominant p16 pathway by overexpressing CDK4 (the natural ligand of p16) in association, or not, with CCND1 is needed for the immortalization of human myoblasts (96–98). As a result, the immortalized DM1 cell lines display potentially unlimited number of divisions while keeping most of the tissue- and disease-specific characteristics. Furthermore, clonal selection leads to homogeneous cell cultures, which allow to provide more consistent and reproducible results. Immortalized cells due to their unlimited lifespan are of special interest when considerably large amounts of cellular material is needed, e.g., for high-throughput screenings (54, 76, 99).

Although the immortalized cell lines present a high value, it is not fully determined yet whether the immortalization process that requires viral transduction for genomic integration and stable expression of hTERT and CDK4 transgenes has any consequences on cellular behavior. Further validations and the use of different cell lines might also be needed to determine whether increasing number of divisions may alter disease- or tissues-characteristics of immortalized cells.

Human Pluripotent Stem Cells (hPSCs)

Cultures of some primary cells types like human neuronal cells constitute a major challenge due to limited biopsies availability and delay of tissue harvesting. The difficulty to obtain material reflecting early stages of the disease process also represents considerable limitations for disease investigations (100). Fortunately, the ability to generate defined cell types from hPSCs offers a unique opportunity to study disease mechanism in a cell-specific manner. hPSCs comprising both embryonic and induced pluripotent stem cells (iPSCs), carry the potential to differentiate them into a wide spectrum of cell types *in vitro*, including the recapitulation of early human embryo development states (101–103). Therefore, they provide an attractive prospect for modeling cell-type-specific disorders.

Embryonic stem cells (hESCs) are isolated from the inner mass of the blastocyst, and can be distinguished by their remarkable long-term proliferative potential along with the ability to differentiate into practically any cell type (**Figure 2**) (102). Although hESCs allow the generation of diverse cell types, up to date most

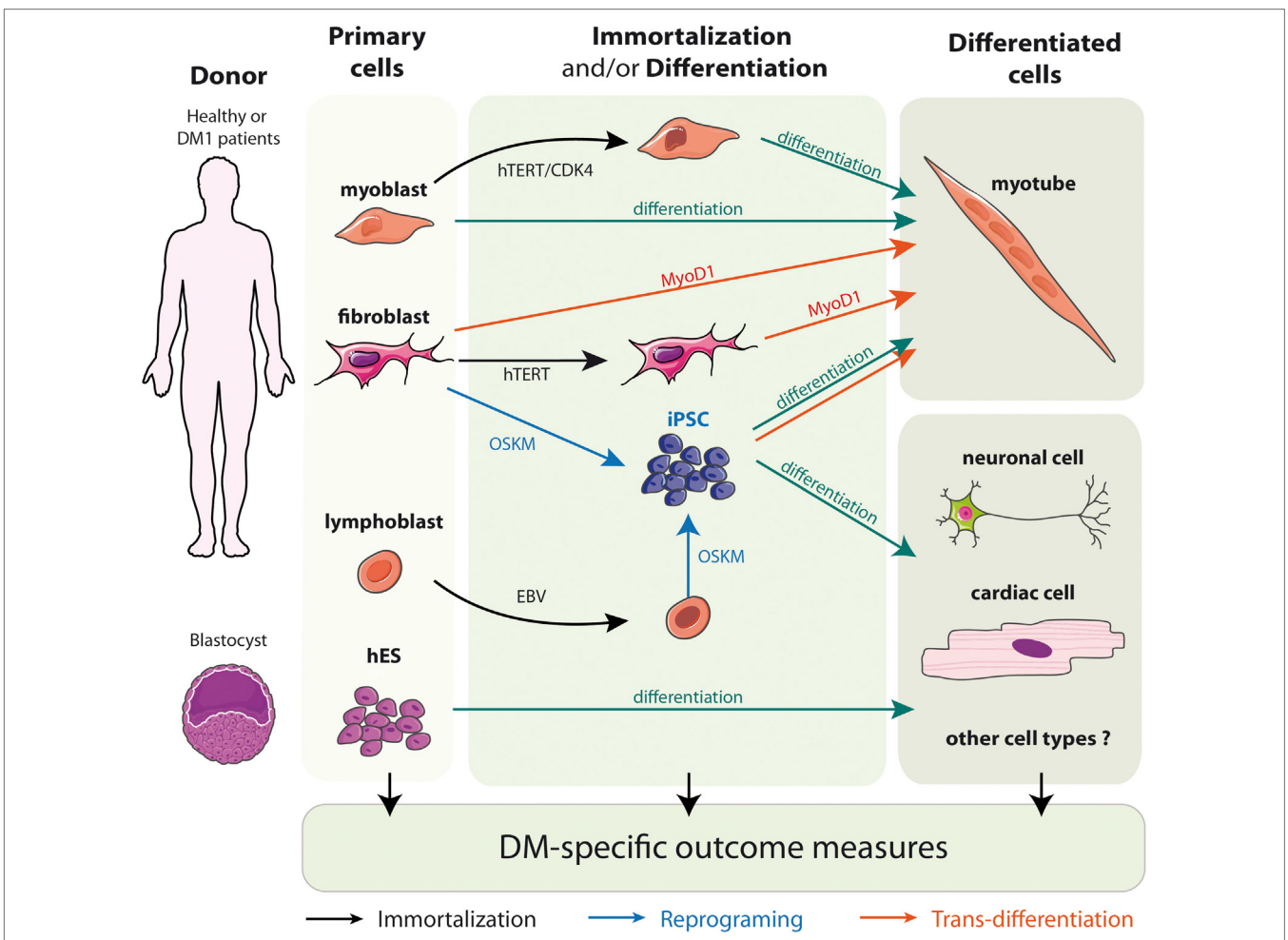

FIGURE 2 | Models of DM1 patients' derived cells.

of the work in the DM1 field has been performed in neural stem cells. Lately, they proved to be additionally useful in identification of new pathways misregulated in the context of DM1 mutation-like disruptions in mTor signaling or defective neuritogenesis (64, 67). However, hESC research raises several ethical issues and has been a subject of controversy over the past 15 years. To obviate the concerns of hESCs, a more recent method of generating patient-specific cells has arisen. It is built on the discovery that somatic cell nuclei can be "reprogrammed" to an embryonic-like state. The whole process begins for obtaining somatic cells, e.g., fibroblasts, from the affected individuals, which are then subjected to delivery of reprogramming factor cocktails (**Figure 2**). Such modified cells are referred to as iPSCs (101). Most importantly, this approach can be applied to human somatic cells, offering a unique opportunity to derive patient-specific cell lines from readily available material (100, 101). Several hiPSc derived from primary fibroblasts of DM patients have already been described (65, 73, 104–107). Interestingly, the ability to reprogram DM1 immortalized lymphoblastic cells carrying 200 CTG repeats into hiPSC also opens the possibility to obtain, in the near future, hiPSC directly from patient's blood samples rather than skin biopsies (106).

Intriguingly, regarding the pluripotent state of the cells, CTG repeats are highly unstable during both reprogramming and subsequent passages, with a more rapid expansion when the initial CTG tract is longer (108). Contradictory to what is observed *in vivo*, several reports have shown CTG repeats instability during culture of undifferentiated DM1-hPSCs but not in differentiated cells, which might be related to some epigenetic differences in these cells (73, 108, 109). In DM1 maternally derived hESC lines, hypermethylation occurs upstream of CTG repeats when repeats number exceeded 300, however, the hypermethylation observed during reprogramming of patients fibroblasts into hiPSCs is not associated with the expansion of CTG repeats (110, 111).

In the past years, a substantial progress in the culture and differentiation technologies associated with hiPSC has been done. Comparing to tissue harvesting, hiPSCs have the advantage of a nearly endless supply. They might be expanded to large quantities and stored for a future expansion or manipulation. Nevertheless, the equivalence of iPS-derived cells to mature *in vivo* cells might vary because they often do not capture the entire mature phenotype. Also, other issues such as homogenous culture of differentiated cells, chromosomal rearrangement during reprogramming and relatively high cost of hPSC maintenance, constitute additional challenges. However, this technology offers a unique opportunity to investigate specific human disease cell types such as neuronal cells or cardiomyocytes for which there are none other or highly limited biological resources.

DM1 CELL MODELS AS A TOOL FOR THE DEVELOPMENT OF THERAPEUTIC APPROACHES

In vitro studies using DM1 cell models contribute also to the development of therapeutic approaches for myotonic dystrophies. Comprehensive studies have been performed in various DM1 cell models to determine and support translational potential of new strategies. Thus, different approaches aim to degrade mutant *DMPK* mRNAs have been tested in DM1 derived cells including gapmer antisense oligonucleotides (ASOs) directed against the CUGexp repeats (55, 112) or the *DMPK* transcript it-self (113) as well as shRNA (114), which have showed significant efficacy in decreasing the level of CUGexp-transcripts. In another hand, CUGexp-steric blocking approaches by using fully modified ASOs or viral-derived antisense RNA proved also to be effective in reversal disease molecular features when tested in DM1 cellular models (77, 78, 112, 115, 116). As a matter of fact, DM1 cells are not only used for therapeutic compounds validation. Indeed this tool is also utilized in screening assays allowing the identification of molecules that either interfere with the abnormal MBNL1:CUGexp interaction such as pentamidine (117, 118) or lomofungin (119), reduce the expression of mutant *DMPK* mRNAs like actinomycin D, modulate splicing changes (72) or affect the behavior of nuclear foci (120–122). Besides, reliable DM1 cell models are essential in the perspective of the recent progress made in genome engineering. TALEN and CRISPR-Cas approaches are being successfully applied in different disease cellular models giving rise to a wide range of possibilities for future therapeutic interventions (65, 70, 95, 123–125). DM1 cell models constitute, therefore, an inescapable source and a flexible platform for thorough studies and validation of disease therapeutics.

CONCLUSION

Through the years, cultured cells showed to be an essential model for both fundamental and translational research on myotonic dystrophy. Despite the fact that cells do not reflect the complexity of a whole organ or body, each cellular model, from the patients' derived cells to more artificial models overexpressing CTG expanded tracts, is suitable for different investigations. They were and are used in many studies addressing various questions related to myotonic dystrophy diseases like mutation lengths, instability, polymorphisms or tissue-specific mechanisms, molecular alterations, and effect of therapeutic approaches. Understanding all of those features paved the way to decipher molecular basics of DM1 and DM2, as both forms share common features, i.e., abnormal expansion of repeated sequences, formation of RNA-positive foci and trans-dominant effect on alternative splicing. Even though DM1 cells served as archetype for DM research, it is noteworthy that some cellular models have been also established for myotonic dystrophy type 2. Further investigations in those cells may emphasize the differences between both DM forms and promote better understanding of their pathological mechanisms. Besides, recent advances in cell-availability and -engineering have given rise to unprecedented experimental opportunities to study disease mechanisms and therapeutic strategies. Late genome engineering tools, with the particular use of emerging development of TALENs and the CRISPR–Cas9 systems, facilitate the next generation of therapeutic interventions and hold a great promise for permanent

genetic corrections (65, 70, 95, 123–125). Furthermore, it opens the door for the development of isogenic cell lines providing a genetically matched "control cells." Alternate possibilities brought by genome editing tools combined with hiPSC technologies promise the generation of novel tissue-specific cell lines opening new horizons for the development of more refined wide-ranging myotonic dystrophy cell models, which will push forward future disease investigations.

AUTHOR CONTRIBUTIONS

MM, AK, FR and DF wrote the review.

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REVIEW
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Small Molecules Which Improve Pathogenesis of Myotonic Dystrophy Type 1

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Myotonic dystrophy type 1 (DM1) is the most common muscular dystrophy in adults for which there is currently no treatment. The pathogenesis of this autosomal dominant disorder is associated with the expansion of CTG repeats in the 3′-UTR of the *DMPK* gene. *DMPK* transcripts with expanded CUG repeats (CUG^{exp}*DMPK*) are retained in the nucleus forming multiple discrete foci, and their presence triggers a cascade of toxic events. Thus far, most research emphasis has been on interactions of CUG^{exp}*DMPK* with the *muscleblind*-like (MBNL) family of splicing factors. These proteins are sequestered by the expanded CUG repeats of *DMPK* RNA leading to their functional depletion. As a consequence, abnormalities in many pathways of RNA metabolism, including alternative splicing, are detected in DM1. To date, *in vitro* and *in vivo* efforts to develop therapeutic strategies for DM1 have mostly been focused on targeting CUG^{exp}*DMPK via* reducing their expression and/or preventing interactions with MBNL1. Antisense oligonucleotides targeted to the CUG repeats in the *DMPK* transcripts are of particular interest due to their potential capacity to discriminate between mutant and normal transcripts. However, a growing number of reports describe alternative strategies using small molecule chemicals acting independently of a direct interaction with CUG^{exp}*DMPK*. In this review, we summarize current knowledge about these chemicals and we describe the beneficial effects they caused in different DM1 experimental models. We also present potential mechanisms of action of these compounds and pathways they affect which could be considered for future therapeutic interventions in DM1.

Keywords: myotonic dystrophy type 1, myotonic dystrophy type 1 pathogenesis, sequestration of *muscleblind*-like 1, antisense oligonucleotides, aberrant alternative splicing, small molecule compounds

INTRODUCTION

Myotonic dystrophy type 1 (DM1) is the most common muscular dystrophy in adults leading to disability and shortened lifespan (1). There is currently no treatment for DM1. The symptoms of this disease include myotonia, muscle weakness and wasting, cardiac conduction defects, diabetes and insulin resistance, and cataracts. DM1 belongs to a larger group of microsatellite disorders associated with expansions of simple repetitive elements within specific genes (2). This autosomal dominant disease is caused by the expansion of CTG repeats in the 3′-UTR of the *DMPK* gene and its pathogenesis is mediated, at least in part, by a toxic RNA *gain-of-function* mechanism. Molecular hallmarks of DM1 cells expressing mutant *DMPK* transcripts (CUG^{exp}*DMPK*) are nuclear RNA foci. Their presence has an adverse effect on host cells leading to a broad spectrum of abnormalities. Recent studies

of the dynamics of CUG repeat foci have revealed that these are unstable, constantly aggregating, and disaggregating structures (3), and *muscleblind*-like (MBNL)1 protein is directly involved in the stochastic process of foci formation. Being associated with CUG^{exp}*DMPK*, the MBNL1 protein has a role in stabilizing RNA aggregates and its downregulation resulted in the decrease of RNA foci accumulation (3). However, MBNL1 depletion does not completely eliminate CUG foci, which suggests involvement of other proteins in mutant transcripts retention. In fact, as demonstrated experimentally, proteins other than MBNL1 may be recruited to the CUG repeat inclusions. Such recruitment may involve limited colocalization, as shown for hnRNP H, hnRNP F, and DDX6 proteins, or it may represent only close association with the RNA foci as shown for SC35 protein (4–7). In addition to the depletion of proteins in CUG RNA foci, DM1 pathogenesis also involves aberrant protein synthesis and/or their altered stability as shown for CUGBP1 protein (8, 9).

Muscleblind-like 1 and CUGBP1 are antagonistic regulators of splicing. MBNL1 is a zinc finger protein which recognizes both RNA sequence (YGCY) and structural elements (hairpins) containing pyrimidine mismatches on either normal splicing substrates or pathogenic mutant repeat RNA (10). As shown *in vitro,* the protein binds selectively to the stem region of expanded CUG RNA, and such interaction is detected in DM1 cells as MBNL1 sequestration and colocalization with mutant CUG repeats (11, 12). On the other hand, CUGBP1 protein binds *in vitro* to single-stranded regions of GU-containing transcripts; however, the protein is not enriched in the RNA foci. In DM1 cells, CUGBP1 becomes hyperphosphorylated, stabilized, and consequently, overexpressed (9). Changes in cellular levels and activities of MBNL1 and CUGBP1 proteins result in the abnormal expression of embryonic splice variants in adult tissues which is one of the molecular hallmarks of DM1 pathogenesis. Besides regulating splicing, both proteins are also involved in mRNA translation, RNA stability, protein secretion, and localization of alternative 3′UTR isoforms (13, 14). Their altered activity in DM1 cells also correlate with changes in signaling pathways of various protein kinases including cyclin-dependent kinases (CDKs), glycogen synthase kinase 3β (GSK3β), AKT, and protein kinase C (PKC) (8, 15, 16).

In vitro and *in vivo* efforts to develop DM1 therapeutic strategies have been mostly aimed at destroying the toxic ribonucleoprotein complexes *via* targeting the mutant CUG^{exp}RNA and/or inhibiting its pathogenic interactions with MBNL1 protein, leading to the generation of several strategies that proved to have a beneficial effect in DM models (17, 18). Thus far, antisense technology that utilizes synthetic siRNAs (19), modified CAG antisense oligonucleotides (20–22), viral vector-mediated expression of hU7-snRNA-(CAG) (23), or a hammerhead RNA (ribozyme) (24) designed to cut CUG repeats, appear effective in DM1 cells and mouse models of the disease. Moreover, morpholino CAG oligonucleotides (25, 26) and several bioactive small molecules (27–34), which are CUG repeat binders, have been reported as potential therapeutic agents for DM1, capable of inhibiting the interactions between expanded CUG RNA and MBNL1 protein.

As our understanding of the pathogenesis of DM1 has grown over the past years, the focus of the research encompassed more molecular events being important for the progression of the disease, e.g., aberrantly spliced genes, RAN translation (35), and deregulation of miRNAs (36). Considering that DM1 is a multisystem disorder, it seems reasonable to target multiple molecules and pathways of the misregulated DM1 apparatus with the aim of developing a beneficial strategy to combat DM1.

Over the past few years, experimental evidence has indicated that, indeed, small molecule chemicals affecting different cellular pathways independently of CUG^{exp}*DMPK* can mitigate some of the molecular hallmarks of DM1 pathogenesis (18). However, it remains elusive, how the molecules alleviate DM1 features. Their efficiency, though, indicates that it is reasonable to search for novel candidate therapeutic targets that will provide new opportunities for future studies aiming to decipher the complex pathomechanism of DM1. In this review, we summarize current knowledge about such molecules, acting independently of direct binding to CUG^{exp}*DMPK,* and we describe beneficial effects they caused in different DM1 experimental models. We also present potential mechanisms of action of these compounds and cellular pathways they affect which could be considered for future therapeutic interventions in DM1.

INHIBITORS OF TRANSCRIPTION

A few compounds that inhibit transcription and alleviate some of the molecular symptoms of DM1 have been identified in recent years. These include pentamidine and its analogs, as well as actinomycin D (ActD) (**Figure 1**) (37–40).

Pentamidine

Pentamidine is an FDA-approved drug currently used to treat patients with *Pneumocystis carinii* infections (pneumonia) in acquired

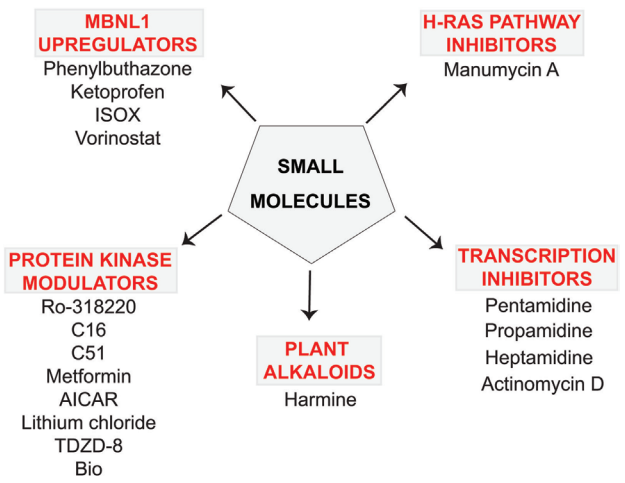


FIGURE 1 | Small molecule compounds alleviating myotonic dystrophy type 1 (DM1) pathogenesis. Small molecule compounds which mitigated DM1 pathogenesis in different experimental systems are shown. The molecules are classified into a few categories depending on their presumable mechanisms of action. All the molecules are believed to act independently of direct interactions with expanded CUG repeats RNA.

immunodeficiency syndrome, as well as patients with *Trypanosomiasis* and *Leishmaniasis* infections (41, 42). Pentamidine is a diamide composed of two phenyl amidine groups joined by a five-carbon methylene linker (**Table 1**). It has been speculated to inhibit the splicing of essential group I introns in *P. carinii*, preventing its growth (43, 44), and it was recently shown to inhibit translation by binding tRNA (45). Pentamidine may also block DNA replication (41), since its structure with DNA showed binding in the minor groove of the double-stranded molecule (46). Therefore, it is likely to interact with many different nucleic acid targets within cells. Interestingly, in DM1 patient's cells and model organisms' pentamidine disrupted CUG RNA foci, released MBNL1 protein, and reversed aberrant alterative splicing of some pre-mRNAs typically misspliced in DM1. Initially, these effects were thought to be caused by binding of the compound to the CUG repeats, because of its ability to bind DNA (40), however, subsequently it was found that its effect on the DM1 molecular phenotype was attributed to either decreased transcription of the CUG transcripts or increased degradation of the toxic RNA (38). So far, the beneficial effects of pentamidine have been shown in HeLa and HEK293 cells transfected with 960 CUG repeats and in model organisms, including the HSA^{LR} mice and a *Drosophila* model expressing 250 CTG repeats (37, 40) (Table S1 in Supplementary Material). Treatment of HeLa cells transiently expressing CUG repeats and transgenic HSA^{LR} mice showed, respectively, a decrease of CUG mRNA expression (and subsequent reduction of CUG RNA foci number), and a reduction of the HSA transcript. Correction of some misspliced pre-mRNAs, i.e., *cTNT* E5 and *INSR* E11 (in HeLa cells) and *Clcn1* E7a and *Serca1* E22 (in HSA mice) was attributed to the liberation of MBNL1 protein from diminished CUG foci and reduction of CUG transcript levels. This suggests that pentamidine does not directly block MBNL1 binding to the repeats and supports the hypothesis that it either inhibits transcription of the CTG repeats or increases the rate of CUG^{exp}RNA degradation. However, in a DM1 *Drosophila* model, a behavioral and molecular improvement which included a minor rescue of cardiac defects reduced CUG foci, and Mbnl1 displacement was not correlated with diminished expression level of CUG repeats mRNA (37) (Table S1 in Supplementary Material). It was suggested that the beneficial effects induced by pentamidine are due to Mbnl1 diffusion and subsequent dispersion of toxic RNA in the nucleus, rather than by inhibition of transcription of the toxic RNA or its degradation.

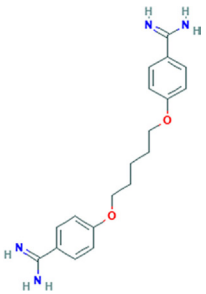
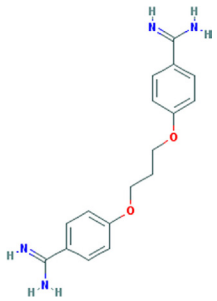
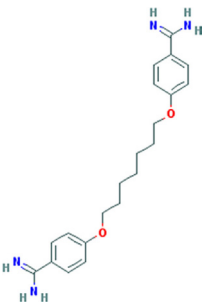
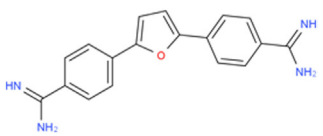
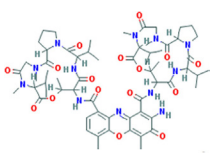
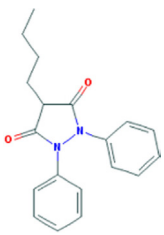
Pentamidine Analogs

The results obtained with pentamidine as an anti-DM1 drug were promising; however, they showed a certain degree of toxicity and thus needed optimization, and the mechanism of action (MoA) needed clarification. To improve the physiochemical properties of pentamidine and to decrease its toxicity, a series of analogs of pentamidine, containing between three to nine methylene carbons, were analyzed for their ability to rescue splicing of transiently transfected minigenes in a HeLa cell model of DM1 and in HSA^{LR} mouse model (Table S1 in Supplementary Material). Two different minigenes, i.e., *cTNT* E5 and *INSR* E11, containing exons that are misspliced in DM1 were tested. Propamidine (methylene linker length of 3) and heptamidine (methylene linker length of 7) (**Table 1**) were identified as the most promising analogs in rescuing missplicing in both DM1 models (38). Although analogs with more methylenes rescued missplicing more efficiently, an increase of the methylene linker length also reduced solubility and increased toxicity. Interestingly, in the HeLa cell model only propamidine was able to reduce CUG repeat transcript levels in a dose-dependent manner (however, not as effectively as pentamidine), whereas no reduction was observed with heptamidine treatment before significant cell death occurred. The *cTNT* splicing was improved to varying degrees with different linker analogs, however, no correction was observed with propamidine at concentrations which were not toxic. Furthermore, the improvement was not dependent on inhibition of CUG transcript expression. Unlike the *cTNT*, all linker analogs partially or fully rescued the missplicing of *INSR* E11 after CUG repeats expression. Different MBNL targets may require different concentrations of the proteins to be properly regulated, which could explain the results found regarding correction of alternative splicing of different genes (47).

Treatment of HSA^{LR} DM1 mice with heptamidine caused reduction of both the mRNA and pre-mRNA levels of the transgene, while in HSA^{SR} mice expressing short CTG repeats, no such reduction was detected. This suggests that the effect was dependent on the presence of extended repeats, rather than the HSA promoter, transgene, or the short repeats. In the HSA^{LR} mouse model, heptamidine completely reversed the missplicing of *Clcn1* E7a, while splicing of *Atp2a1* E22 was rescued partially and at lower concentrations than pentamidine. In addition, heptamidine caused a rapid (after 1 week) and significant reduction of myotonia, as well as diminished levels of HSA^{LR} transcript (Table S1 in Supplementary Material). Nevertheless, it showed high toxicity in the mouse model. However, variation of the linker length of pentamidine resulted in significant improvements and is encouraging to develop a therapy for DM1. Reduction of the CUG transcript in *in vitro* transcription assays as well as in both DM1 models, i.e., HeLa cells and HSA^{LR} mice, as well as reduced formation of nuclear foci, suggest that pentamidine and its analogs most likely inhibit transcription of CTG*CAG repeat DNA. However, other possible mechanisms cannot be ruled out as pentamidine is a DNA-binding molecule and may affect expression of other genes, some of which may be regulators of alternative splicing.

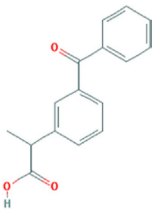
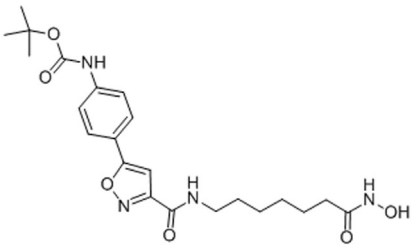
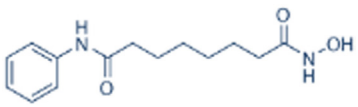
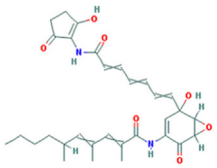
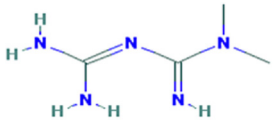
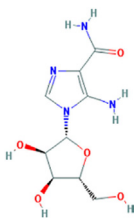
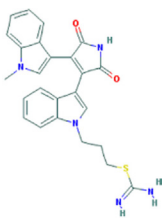
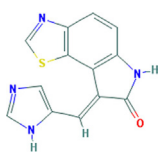
Further modifications of pentamidine and heptamidine to modify their size, solubility, degrees of freedom, number of hydrogen bond donors, and hydrophobicity led to the generation of new promising compounds. These molecules were tested for their ability to correct splicing defects in cellular and transgenic mouse models of DM1, as well as their toxicity relative to the parental compounds (48). Three different series of molecules varying in amidine substitution, central linker length, and planarity were synthesized. Compound 8, a molecule with an un-substituted propyl chain, was able to rescue missplicing in the DM1 HeLa cell model with no toxicity at the range of concentrations used, but it was half as effective as pentamidine. Modification of the planarity produced a linear molecule, compound 12, and a molecule with a concave shape, compound 13 (**Table 1**). Both compounds rescued missplicing similarly to pentamidine and they were not toxic over the range of concentrations needed for

TABLE 1 | Molecular formula and chemical structure of small molecule compounds which do not directly target expanded CUG repeats and alleviate pathogenesis of myotonic dystrophy type 1.

Name	Molecular formula	Structure	Potential mechanism of action
Pentamidine	C19H24N4O2		Inhibition of transcription
Propamidine	C17H20N4O2		
Heptamidine	C21H28N4O2		
Compound 13	C18H16N4O		
Actinomycin D	C62H86N12O16		
Phenylbuthazone	C19H20N2O2		Upregultion of *muscleblind*-like 1

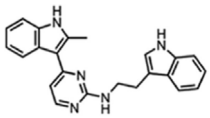
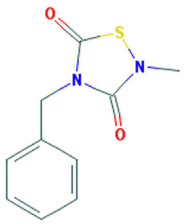
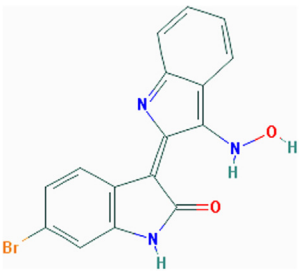
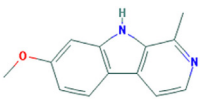
(*Continued*)

TABLE 1 | Continued

Name	Molecular formula	Structure	Potential mechanism of action
Ketoprofen	C16H14O3		
ISOX	C22H30N4O6		
Vorinostat	C14H20N2O3		
Manumycin A	C31H38N2O7		Inhibition of H-RAS pathway
Metformin	C4H11N5		Modulation of protein kinases
AICAR	C9H14N4O5		
Ro 31-8220	C25H23N5O2S		
C16	C13H8N4OS		

(*Continued*)

TABLE 1 | Continued

Name	Molecular formula	Structure	Potential mechanism of action
C51	C23H21N5		
Lithium chloride	LiCl		
TDZD-8	C10H10N2O2S		
6-Bromoindirubin-3′-oxime	C16H10BrN3O2		
Harmine	C13H12N2O		Undetermined

the rescue, unlike pentamidine. Compound 12 affected splicing in a DM-independent manner in a model without CUG expanded repeats, suggesting a modulation of splicing through a mechanism beyond targeting the CUG repeats. On the contrary, compound 13 only rescued alternative splicing in the presence of CUG repeats. Furthermore, it reduced foci in the DM1 HeLa cell model and partially rescued misplicing of *Clcn1* E7a and *Atp2a1* E22 in the HSA^{LR} mouse model at a similar level observed with heptamidine, but without the associated toxicity (39).

The MoA of pentamidine has been previously proposed to be inhibition of transcription through binding to the CTG repeats or reduction of the stability of the transcript. Nonetheless, binding to the CUG repeats and therefore displacement of MBNL1 proteins, especially in the case of the analogs generated has not been completely ruled out, and further work is required to clarify the MoA of the diamines as anti-DM1 drugs.

Actinomycin D

Actinomycin D (**Table 1**) is a polypeptide antibiotic which forms a stable complex with double-stranded DNA, inhibiting DNA-primed RNA synthesis and causing single-stranded breaks in DNA, therefore stopping the proliferation of cells (49, 50). ActD is an FDA-approved anti-cancer drug with activity to inhibit global transcription. More recently, this antibiotic was shown to improve the DM1 molecular phenotype (39). ActD does not bind to CUG repeat RNA *in vitro*, however, low concentrations, insufficient to affect global transcription, triggered a significant reduction of expression of the expanded CUG repeat RNA in HeLa cells and in DM1 fibroblasts, by 50–70% and by 44–60%, respectively. At effective dosages, ActD was mildly toxic to HeLa cells causing reduction of nuclear CUG^{exp}RNA foci by 50% and releasing MBNL1 from the foci. In HSA^{LR} mice, the molecule specifically reduced mRNA levels of the repeat-containing HSA transgene and completely rescued the aberrant splicing of *Clcn1* E7a, whereas partially corrected splicing of a few other genes, i.e., *Atp2a1* E22, *Mbnl1* E7, *Vps39* E3, *Nfix* E7, and *Ldb3* E11. Importantly, at the range of dose used these changes were not correlated with global inhibition of transcription (Table S1 in Supplementary Material). The MoA of ActD in DM1 is yet to be determined. However, its effects produced in the disease models suggest inhibition of transcription of the CUG^{exp}RNA by binding to the CTG repeats in DNA and blocking the RNA polymerase. Such mechanism is feasible since it is known that ActD intercalates into GC-rich sequences, stabilizing topoisomerase-I DNA complexes and preventing RNA polymerase progression (51).

COMPOUNDS UPREGULATING MBNL1

Overexpression of MBNL1 protein has been shown to alleviate pathogenesis of DM1 by reversal of aberrant alternative splicing

and rescue of myotonia (52). Accordingly, small molecules which upregulate expression of the splicing factor would have a potential of becoming therapeutic molecules for DM1. Two compounds with such capacity have been identified by Chen et al. (53). Phenylbutazone (PBZ) and ketoprofen (**Table 1**) belong to nonsteroidal anti-inflammatory drugs (NSAIDs) which are used to reduce pain and fever, prevent blood clots, and in higher doses, decrease inflammation. NSAIDs inhibit the activity of cyclooxygenase enzymes (COX-1 and/or COX-2). These enzymes participate in the synthesis of key biological mediators in the cells, such as prostaglandins which are involved in inflammation, and thromboxanes which are involved in blood clotting (54, 55).

It was shown that PBZ enhances MBNL1 expression in proliferating and differentiating C2C12 cells in a dose-dependent manner (53) (Table S1 in Supplementary Material). Consistent with this result, when analyzed in HSA^{LR} DM1 mice PBZ elevated the expression of *Mbnl1 mRNA* and protein in tibialis anterior and in quadriceps muscles. Interestingly, the expression of CUGBP1 remained unchanged after the treatment with PBZ. Consequently, the treated mice showed partial rescue of aberrant splicing of MBNL1-dependent exons, such as *Clcn1* E7a, *Nfix* E7, and *Rpn2* E17, whereas no splicing correction was found for CUGBP1-regulated exons. Amelioration of the molecular features in HSA^{LR} mice was further confirmed in behavioral and histological tests and the mice had an increase of grip strength and decrease in the number of muscle fibers with central nuclei (53). Although PBZ elevated Mbnl1 expression levels in HSA mouse muscles, colocalization of the protein with CUG RNA foci was markedly attenuated by its treatment. This result suggests that PBZ inhibits the interaction between CUG RNA foci and MBNL1 and reduces the ratio of MBNL1 in the mutant transcript. The precise MoA of PBZ in DM1 remains unclear and it is speculated that it may not be limited to the liberation of MBNL1 proteins from CUG^{exp}RNA. The observed upregulation of *MBNL1* mRNA was attributed to demethylation of its intron 1, making this region to act as an enhancer of transcription (53).

Ketoprofen is another NSAID which was able to upregulate MBNL1 levels in C2C12 cells (53). In a *Drosophila* model of DM1 expressing 480 interrupted CUG repeats the compound suppressed CUG-mediated lethality (56). The MoA of ketoprofen in DM1 has not yet been elucidated.

More recently, a flow cytometry-based screen led to identification of small compounds that upregulated MBNL1 and partially rescued splicing defects in DM1 patient-derived fibroblasts (57). Using engineered HeLa cells containing a ZsGreen fluorescent tag in the N-terminus of the MBNL1 sequence, the HDAC inhibitors ISOX, and Vorinostat (**Table 1**) were found to produce a 2- and 1.8-fold increase of the ZsGreen-MBNL1 signal, respectively (57). Treatment of either normal or DM1 fibroblasts with ISOX and Vorinostat produced a significant increase of MBNL1 levels, and partially rescued the splicing of *SERCA1* e22 and *INSR* e11 in both cell lines (Table S1 in Supplementary Material). Since ISOX and Vorinostat are HDAC inhibitors, they may affect expression of *SERCA1*, *INSR*, and *DMPK* mRNAs. However, as shown by Zhang and coauthors, treatment of normal and DM1 fibroblasts with these molecules caused no significant changes in the genes levels. The MoA of ISOX and Vorinostat is not fully clear, but inhibition of HDAC appears to have a role in modulating MBNL1 levels, and the effects might be caused by inhibition of several HDACs. ISOX inhibits HDAC6 at low concentrations, but also inhibits HDAC1 and other HDACs at higher concentrations. Vorinostat is an FDA-approved HDAC inhibitor for the treatment of cutaneous T cell lymphoma, and inhibits class I and class II HDACs, altering gene transcription and causing cell cycle arrest. Nonetheless, in the light of these recent results, modulation of MBNL1 through mechanisms other than inhibition of HDAC cannot be completely ruled out (57).

INHIBITORS OF H-RAS PATHWAY

The Ras family includes three members: H-Ras, K-Ras, and N-Ras. They play roles in a large number of biological processes including cell morphology, survival, apoptosis, gene expression, and alternative splicing regulation (58). Posttranslational modifications of Ras proteins lead to their activation and these modifications include farnesylation of H-Ras, as well as farnesylation and geranylgeranylation of K-Ras and N-Ras (59). Manumycin A (**Table 1**) is an FDA-approved antibiotic that acts as a potent and selective farnesyltransferase (FTase) inhibitor (60). By inhibiting FTase, manumycin A prevents activation of H-Ras protein but has no effect on K-Ras and N-Ras. It has been reported that some of DM1 features can be alleviated by exposure to manumycin A (**Figure 2**) (61). Treatment of HSA^{LR} DM1 mice with the compound led to correction of *Clcn1* E7a splicing, what was linked to the inhibition of H-Ras activity since siRNA knockdown of endogenous H-Ras protein recapitulated improvement of the splicing. Such effect was not detected when the two other Ras proteins were downregulated. Although skeletal muscle injections with manumycin A corrected aberrant splicing of *Clcn1* in DM1 mice, splicing of two other genes, *Serca1* E22 and *m-Titin* Mex5, was not altered (61). Importantly, in this experimental model of DM1, manumycin A did not alter expression of Mbnl1 and Cugbp1, which are involved in splicing regulation of *Clcn1* E7a, *Serca1* E22, and *m-Titin* Mex5 (Table S1 in Supplementary Material). Thus, it was concluded that the effect of manumycin A on aberrant splicing was independent of these two splicing factors. Therefore, it is possible that manumycin A, which acts as a Ras FTase inhibitor, triggers alterations in H-Ras signaling. This may influence a *trans*-acting factor(s) other than MBNL1 and CUGBP1 involved in alternative splicing and may contribute to chloride channel splicing. However, a category of *trans*-acting factor(s) affected remains unknown.

MODULATORS OF PROTEIN KINASES

Protein Kinase Inhibitors

Pathogenesis of DM1 has been linked to disrupted protein kinase signaling pathways. Altered expression and nonspecific activation have been shown for PKC, Src family kinases, CDKs, GSK3β, and protein kinase AKT (8, 15, 16). Current studies in DM1 with the use of small molecule inhibitors of protein kinases have shed light on a potential alternative strategy in DM1 therapeutic intervention. Screening of several libraries of small molecule compounds,

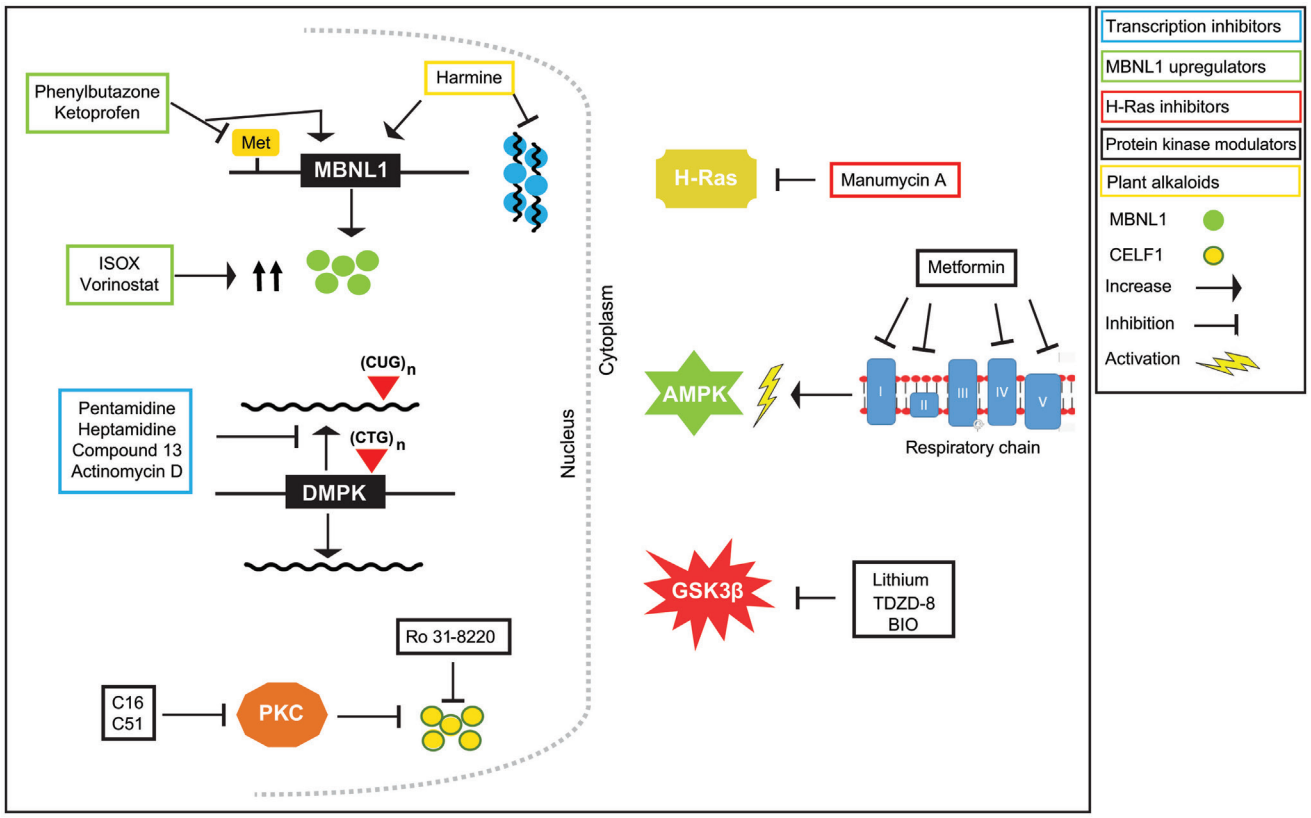


FIGURE 2 | Therapeutic effects of small molecules on myotonic dystrophy type 1 (DM1) pathogenesis. DM1 is characterized by the presence of RNA foci which are aggregations of the mutant CUG^{exp} transcript with *muscleblind*-like (MBNL)1 and other proteins. CUGBP1 is not sequestered by foci, but it is upregulated. The imbalance of these two alternative splicing proteins causes the aberrant alternative splicing of many pre-mRNAs. Treatment of DM1 cells and model organisms with small molecules that target the DNA and/or affect proteins involved in the DM1 pathogenesis can lead to beneficial effects, such as inhibition of transcription of the mutant transcript, or its degradation, release of MBNL1 protein from RNA foci, downregulation of CUGBP1 protein, and ultimately the correction of the aberrant splicing.

including phosphatase and kinase inhibitors, using a medium throughput phenotypic assay by Ketley et al. led to the identification of such molecules (62). Based on the identification of nuclear foci in DM1 cells using *in situ* hybridization and high-content imaging, Ro 31-8220 (**Table 1**) was identified as a compound of potential therapeutic benefit in DM1. The compound eliminated nuclear CUG^{exp}RNA foci, reduced MBNL1 protein in the nucleus, affected *SERCA1* E22 alternative splicing, and decreased steady-state levels of CUGBP1 protein (**Figure 2**). Ro 31-8220 was previously identified as a PKC inhibitor and seen to affect the hyperphosphorylation of CUGBP1 and ameliorate the cardiac phenotype in a DM1 mouse model (8, 63). Nonetheless, Ketley et al. demonstrated that Ro 31-8220 acts independently of PKC on DM1 pathomechanism, suggesting the involvement of other kinases. Although the MoA of Ro-31-8220 still requires further investigation, the compound is likely to work independently of CUG repeat RNA binding.

Another study has identified two compounds, C16, an imidazolo-oxindole inhibitor and C51, a pyrimidine-based inhibitor (**Table 1**), of kinase inhibitory activity with the potential to alleviate DM1 molecular phenotype (64). Previous studies have described these two compounds as ATP-site directed PKR inhibitors (30, 65); however, activity of C16 against kinases other than PKR has also been reported (66). C16 has been successfully used *in vitro* and *in vivo* as an effective and versatile neuroprotective agent and shown to have potential value in the treatment of neurodegenerative diseases (66–69). Results by Wojciechowska et al. demonstrate that C16 and C51 may have therapeutic potential in patients with DM1 as well. These compounds produced a redistribution of the MBNL1 protein sequestered in CUG^{exp}RNA foci and a reduction of the steady-state levels of CUGBP1. These actions were accompanied by correction of the aberrant alternative splicing of MBNL1-dependent (*SERCA1* E22, *DMD* E78, *MBNL1* E7, and *LDB3* E7) and CUGBP1-dependent (*ITGA6* E24, *MTMR3* E16, and *SORBS1* E6) pre-mRNA targets, shifting the patterns of spicing toward those observed in non-DM cells. However, despite causing the nuclear CUG^{exp}RNA foci to become less abundant, as determined by *in situ* RNA hybridization and immunocytochemistry for MBNL1 protein, the compounds did not eliminate foci completely.

GSK3β Inhibitors

Glycogen synthase kinase 3β has been seen to have a role in DM1 pathogenesis (16). This kinase phosphorylates cyclin D3 at T283, which triggers degradation of the cyclin. Cyclin D3 associates with CDK4 and the resulting complex phosphorylates CUGBP1 at S302 and regulates the translational activity of the protein. CUGBP1 regulates translation of proteins important for skeletal muscle development, thus its normal translational activity is required for a proper myogenesis. In muscle biopsies of DM1 patients, GSK3β was found to be increased, causing a degradation of cyclin D3 and a reduced phosphorylation of CUGBP1 at S302, what was linked to delayed myogenesis. The presence of CUG expanded repeats appears to increase the stability of GSK3β by causing the autophosphorylation of the protein at Y216 (16, 70). Altogether, these observations point at GSK3β as a potential therapeutic target in DM1. Indeed, treatment of HSA^{LR} mice with two GSK3β inhibitors, lithium and TDZD-8 (thiadiazolidine) (**Table 1**), reduced the levels of GSK3β in skeletal muscle and normalized the levels of cyclin D3, restoring CUGBP1 translational function. These changes improved skeletal muscle strength in the mice and reduced myotonia, suggesting that correction of GSK3β may have a beneficial effect on myofiber regeneration (16).

Another GSK3β inhibitor, 6-bromoindirubin-3′-oxime (BIO) (**Table 1**), was used to treat young HSA^{LR} mice, resulting in recovery of the grip strength to near-normal levels and the effect was maintained for several months after completion of the treatment. In addition, the levels of GSK3β and cyclin D3 in 12-month-old mice previously treated with BIO were similar to those in control mice, and the translational activity of CUGBP1 was corrected. Results from this study suggest that inhibition of GSK3β in young mice is sufficient to maintain corrected levels of the GSK3β-cyclin D3-CUGBP1 pathway and nearly normal muscle health over a long period of time after completion of the treatment (71).

Activators of AMP-Activated Protein Kinase (AMPK)

Metformin (**Table 1**) is an FDA-approved antidiabetic drug and was earlier reported to improve hyperglycemia through increased insulin-independent glucose uptake in peripheral muscles of DM1 patients (72). Most recently, metformin was investigated in *in vitro* DM1 models of human embryonic stem cells and in primary myoblasts derived from patients (73). The drug appeared to modify the alternative splicing of a subset of genes associated with DM1 in these cell models, as it partially rescued the aberrant splicing of *INSR* E11, *CLCN1* E7a, *TNNT2* E5, *ATP2A1* E22, and *DMD* E78. The effect on the modification of the alternative splicing has been linked to the inhibition of the complex I of the respiratory chain, which in turn raises the intracellular AMP/ATP ratio, which triggers the activation of AMPK. The role of AMPK activation in alternative RNA splicing was tested in DM1 myogenic progenitor cells and in myoblasts with the AMPK activator AICAR [5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside, Acadesine, N^1-(β-D-ribofuranosyl)-5-aminoimidazole-4-carboxamide] (73). Treatment of DM1 cells promoted changes in similar subsets of pre-mRNAs as found for metformin. However, AICAR did not modulate the *INSR* E11 splicing in the cells used. Thus, these results suggest that activation of AMPK is partially involved in alternative splicing modulation, but metformin appears to trigger an additional molecular pathway. Interestingly, metformin decreases tyrosine kinase receptor signaling (73). The tyrosine kinase receptors include the epidermal growth factor receptor, the signaling pathway which controls *INSR* E11 inclusion *via* the inhibition of *hnRNPA1* and *hnRNPA2B1* expression (74, 75). This could explain the effect of metformin on the alternative splicing of *INSR*. The MoA behind other splicing events modulated by metformin but not by AMPK activation remains unclear.

SMALL MOLECULES OF NATURAL ORIGIN

Many small molecules have been synthesized as potential therapies for DM. However, only a few studies have reported utilization of molecules of natural origin (40, 76). Recently, a set of plant-derived alkaloids was identified as small molecules with an anti-DM1 effect (77). Using a novel CUG_{78}–MBNL1 complex inhibition assay, a collection of isolated natural compounds and extracts from plants and fungal strains was screened. The bioactivity of the compounds was investigated in human DM1 cells and HSA^{LR} mice resulting in the identification of several alkaloids, including carboline harmine and isoquinoline berberine, which ameliorated certain aspects of the DM1 pathology in these models.

Aromatic alkaloids can interact with RNA, and indeed berberine and harmine (**Table 1**) have been reported to bind RNA structures (78, 79). In DM1 myoblasts, harmine reduced foci, nevertheless it did not improve the histology in gastrocnemius muscle of the HSA^{LR} mice, as the percentage of fibers with internalized nuclei was not altered by the treatment of the mice with the compound (77). However, although harmine increased the levels of MBNL1 in DM1 myoblasts and enhanced MBNL1-dependent alternative splicing of *cTNT* E5, *INSR* E11, and *Clcn1* E7a (Table S1 in Supplementary Material), a similar effect was found in wild-type myoblasts, suggesting that inhibition of the CUG–MBNL1 complex is not the primary MoA of this alkaloid. Consequently, it was suggested that harmine acts through another mechanism that causes the increase of MBNL1 levels and the amelioration of the spliceopathy. Despite the side effects, harmine represents an interesting small molecule that could be optimized by chemical modifications to become a potential DM1 therapy.

CONCLUSION

Myotonic dystrophy type 1 is a life-shortening, debilitating disorder for which there is currently no treatment. Pathogenesis is associated with nuclear retention of mutant DMPK mRNA which attract or is attracted by various proteins. Experimental data suggest that the formation of riboprotein complexes is a necessary trigger for DM1 pathogenesis. Thus, compounds which reduce such inclusions would be therapeutically beneficial. Over the past few years, many efforts have been focused on the synthesis of small molecule chemicals specifically recognizing mutated CUG

repeats and either cutting the toxic RNA or blocking their interactions with relevant proteins (19, 22, 26). In both cases, treatment improved DM1 molecular and behavioral features including fewer CUG^{exp}RNA foci, liberation of MBNL1 protein, rescue of aberrant alternative splicing, and muscle pathology correction. Interestingly, similar improvements have also been observed with other small molecules affecting the DM1 mutation indirectly (38, 48, 62). Although there are many questions concerning the mode of action of these chemicals, *in vitro* and *in vivo* efficacy underline the notion that these molecules can have therapeutic benefit in DM1. Furthermore, several of the described molecules are FDA-approved drugs, potentially offering an opportunity of repositioning.

The development of therapeutic approaches based on small molecules has several advantages, including lower costs with ease of manufacturing, ease of management of the therapy, with the possibility to rapidly interrupting the treatment in case of toxicity, ease of administration and tissue delivery, opportunities of repositioning, and most notably, that the pharmaceutical industry has decades of experience in refining and improving potentially useful compounds *via* conventional medicinal chemistry-based approaches. Such efforts may prove fruitful for DM1.

AUTHOR CONTRIBUTIONS

Conception and design and wrote the main manuscript text: ML-M, MW, and JDB. Prepared tables and figures: ML-M and MW. All authors reviewed the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fneur.2018.00349/full#supplementary-material.

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