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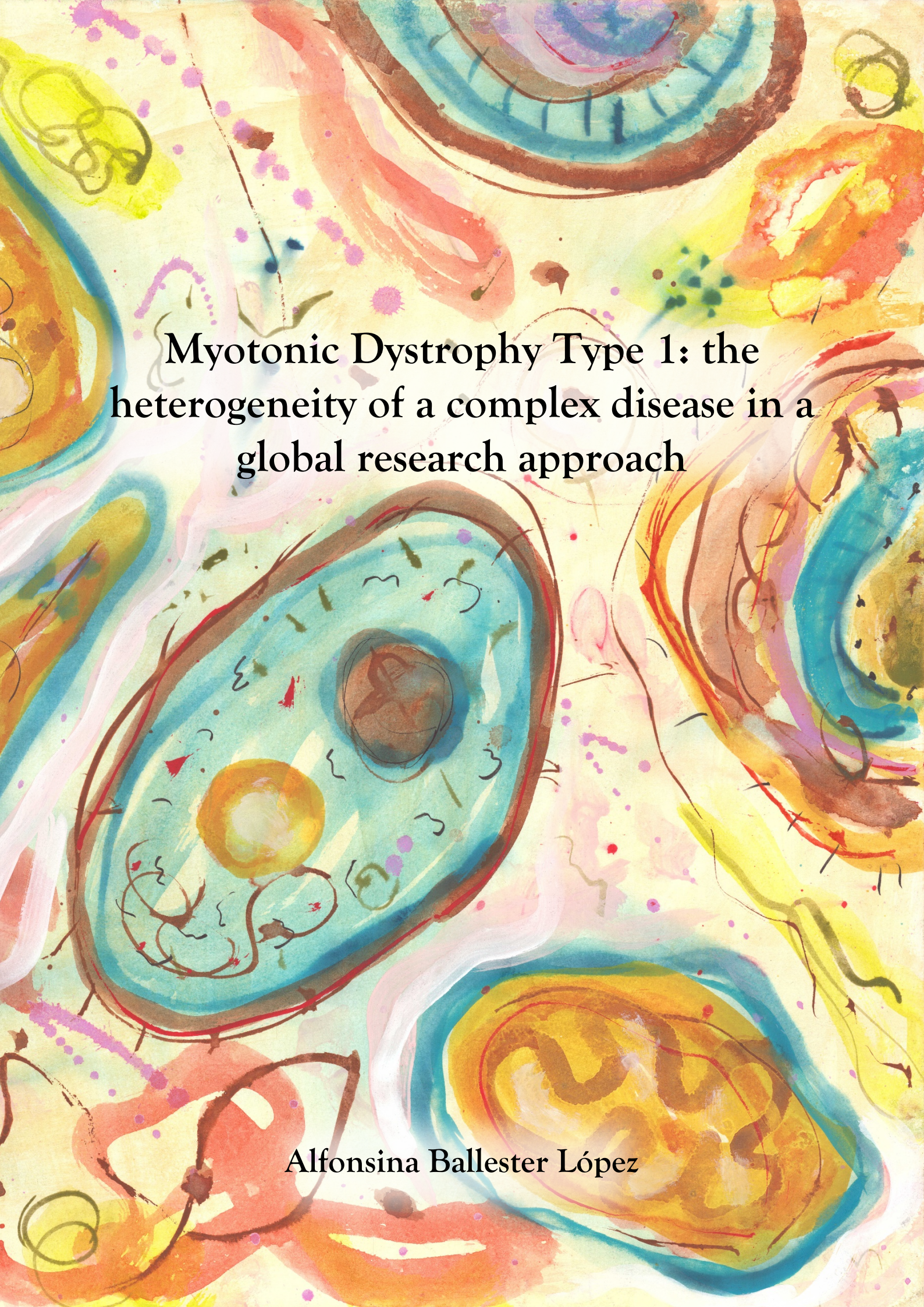

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PhD Thesis

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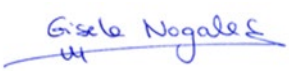
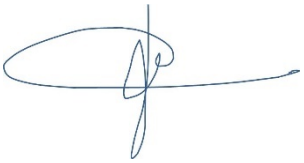
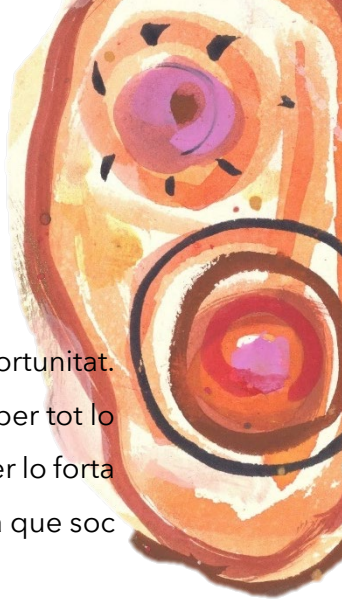




Cover and illustrations made by Antoni Jesús Ballester Forteza

A ells, es meus dos pilars, Papá i Mamá



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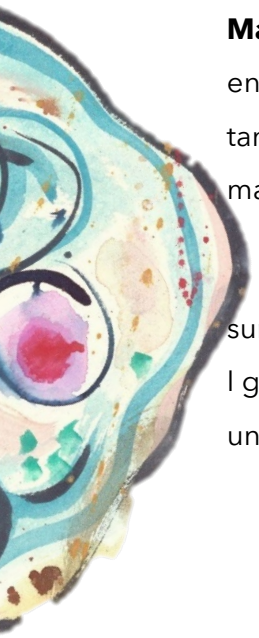

Sé que em deixo a gent, tinc al cap a moltes persones que el seus noms no surten aquí, i que també han format part de tots aquests anys. Gràcies, gràcies a tots. I gràcies a TU, tot i que el teu nom no hi sigui, gràcies per llegir-me i, d'una manera o una altra, formar part de tot això.

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This is just the beginning,

I'm ready to fly

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Abbreviations

2′-cEt – Constrained Ethyl Nucleotide

3D – Three-Dimensional

AMPK – Adenosine Monophosphate activated Protein Kinase

ASO – Antisense Oligonucleotide

ATP2A1 – Sarcoplasmic/Endoplasmic Reticulum Ca^{2+}-ATPase 1

BNA – Bridged Nucleic Acid

CAG – Cytosine-Adenine-Guanine

CCG – Cytosine-Cytosine-Guanine

CGG – Cytosine-Guanine-Guanine

ClC-1 – Muscle Chloride Channel

CTC – Cytosine-Thymine-Cytosine

CTG – Cytosine-Thymine-Guanine

cTNT – Cardiac Troponin T

CUG – Cytosine-Uracil-Guanine

CUGBP1 – CUG-Binding Protein 1

DM1 – Myotonic Dystrophy Type 1

DM300 – Mouse model carrying from 300 to 600 CTG repeats

DMPK – *Dystrophia Myotonica*-Protein Kinase

DMSXL – Mouse model carrying from 1000 to 1800 CTG repeats

DMWD – *Dystrophia Myotonica* WD repeat-containing

GC – Guanine-Cytosine

GGC – Guanine-Guanine-Cytosine

GSK3ß – Glycogen Synthase Kinase 3 Beta

HPE-PCR – Heat Pulse Extension-PCR

HSA^{LR} – Human Skeletal Actin Long Repeat length

IR – Insulin Receptor

LNA – Locked Nucleic Acid

LPCR-SB – Long PCR-Southern Blot

MBNL – Muscleblind-Like

MIRS – Muscular Impairment Rating Scale

MOE – 2′-O-Methoxyethyl

MRC – Medical Research Council

mRNA – Messenger Ribonucleic Acid

mRS – Modified Rankin Scale

mTOR – Mammalian Target of Rapamycin

OMe – 2′-O-Methyl

PBMCs - Peripheral Blood Mononuclear Cells

PCR – Polymerase Chain Reaction

PKC – Protein Kinase C

PMO – Phosphorodiamidate Morpholino

PNA – Peptide Nucleic Acid

PS – Phosphorothioate

SIX5 – Six Homebox 5

SP-PCR – Small Pool-PCR

TP-PCR – Tripled-repeat primed PCR

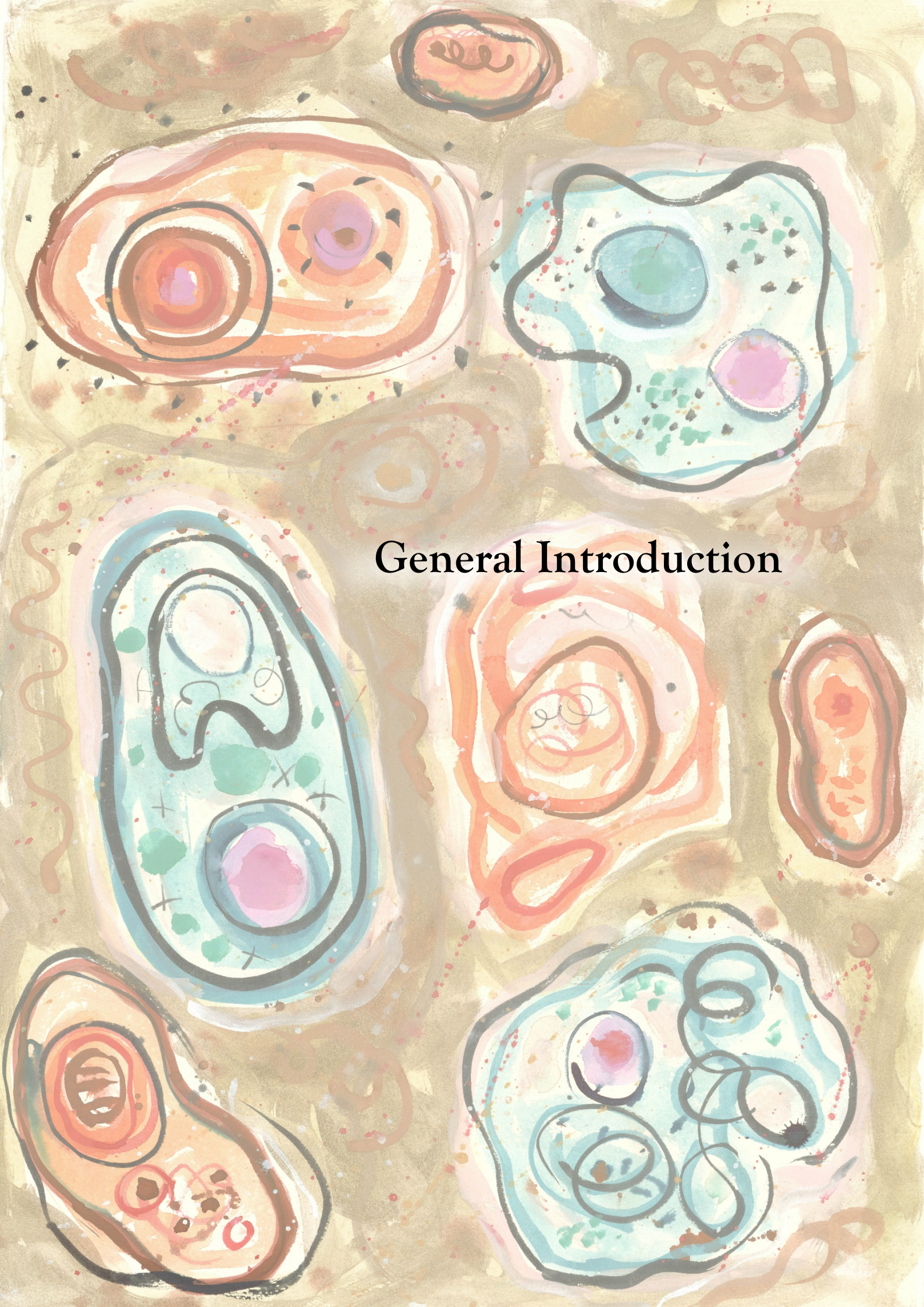
UTR – Untranslated Region

List of synonyms

CTG expansion; CTG repeats; CTG repeat track; CTG repeat expansion; CTG triplets; CTG track; CTG fragment.

CTGs; CTG expansion size; CTG size; CTG length track; CTG repeat length; CTG expansion length; CTG repeat number; CTG number; CTG repeat size.

Interruptions; variant repeats.



General Introduction

1. A brief story about Myotonic Dystrophy Type 1

Myotonic Dystrophy Type 1 [DM1 (MIM: 160900)] is a neuromuscular disorder that was first described by Hans Steinert in 1909 with his work titled "About the clinical and anatomical appearance of muscular atrophy in patients with myotonia", which he called "Dystrophien Myotoniker" [1]. However, the genetic defect responsible for DM1 was not discovered until 1992, and it was found to be an autosomal dominant disorder caused by a trinucleotide expansion in the *dystrophia myotonica*-protein kinase (*DMPK*) gene (DMPK; MIM 605377) [2]. Nowadays, DM1 is the most common muscle dystrophy in adults, with an overall estimated worldwide prevalence of 1 in 2500 individuals [3,4]. Although it is characterized by skeletal muscle distal weakness and myotonia, DM1 is not purely a muscle disease, but a multisystemic disorder instead, showing a high variability of symptoms in between patients such as heart conduction defects, respiratory problems, cataracts, and gastrointestinal disorders [4]. The severity in the patients also varies widely, ranging from asymptomatic or only mildly affected adults in old age, to severely affected neonates. Thus, the age of disease onset in DM1 also varies, from birth to old age.

Overall, DM1 is a challenging disease, with a wide clinical spectrum, highly complex genetics, and many pathological molecular mechanisms.

2. Clinical characteristics of DM1 patients

The phenotype complexity in DM1 is divided into five main clinical categories: congenital, infantile, juvenile, adult-onset and late-onset forms [5]. The clinical heterogeneity in DM1 creates no clear distinctions for some specific clinical features [6]; however, in each of these five forms, there is a distinction from each other based on the prevalence of the main symptoms and the onset profile [7–9].

Congenital DM1

Congenital DM1 comprises DM1 patients with fetal-onset until one month of life [9], and are the most severely affected by the disease. Before birth, congenital DM1 is often presented as polyhydramnios and reduced fetal movements [10]. After

delivery, the main general features are severe weakness (which makes suckling and swallowing difficult), cognitive impairment, hypotonia and early respiratory insufficiency [4,9,11]. In the congenital DM1, respiratory failure is highly present and may require mechanical ventilation. However, children ventilated for longer than three months have a 30% of mortality in the first year of life [12]. Congenital patients who reach adulthood rarely live beyond the age of 40 [13].

Infantile form

The clinical onset of infantile DM1 patients ranges from one month to 10 years old [9]. Children with DM1 are able to walk [10] and can have a progressive myopathy and milder myotonia; although those are muscle features that are highly seen in the classical form of DM1 [14]. However, these patients have severe cognitive impairment (compromising the learning capacities) [15], facial weakness, gastrointestinal problems [8] and cardiac conduction defects [16]. Patients with childhood DM1 survive into adulthood, however the determination of the natural history is related to the advances in supportive care [17].

Juvenile form

The disease onset of juvenile DM1 form ranges between 10 and 20 years [9]. Although infantile and juvenile DM1 forms share and overlap some features, juvenile patients express less marked cognitive impairment than the infantile form of DM1 and have earlier endocrine disorders [9,18]. The juvenile form has a higher prevalence of myotonia over the other forms [9]. In addition, these patients have earlier gastrointestinal problems, cardiac issues, respiratory insufficiency and muscle defects compared to the classical adult DM1 form [9].

Classical adult form

Around 75% of patients develop the classical adult form, which has an onset between 20 and 40 years [9]. The predominant symptoms in the classical form are distal muscle weakness and myotonia [4]. Weakness is also present in the muscles of the face, and together with the wasting of the facial and masticatory muscles, leads to a typical facial expression characterized by ptosis and flaccid dysarthria [19]. The weakness and myotonia of these patients cause swallowing difficulties and lead to

aspiration pneumonia [20,21]. Other gastrointestinal problems derived from myotonia and smooth muscle loss are slow gastric emptying and constipation [22]. Classical DM1 patients also have respiratory failure and cardiac conduction defects, which contribute significantly to the disease mortality with a high percentage of sudden death [23–25]. The intellectual deficits in the adult forms are not as major as they are in congenital and infantile forms [11,26]. However, an avoidant, obsessive-compulsive and passive-aggressive personality features have been reported [27], as well as anxiety and depression, which are common among these patients [28]. An excess of daytime sleepiness is also extremely common [29]. Endocrine abnormalities are also present in classical adult-onset, including disturbances in the pancreas, thyroid, hypothalamus and gonads, affecting the fertility of the patients [30], as well as insulin insensitivity [31]. These patients can also develop skin alterations such as pilomatrixoma and epitheliomas as well as early frontal balding, which is more common in males than in females [32,33]. DM1 patients usually develop cataracts [34] as well. Moreover, epidemiologic studies have shown that DM1 is associated with a higher risk of cancer, especially involving the thyroid gland, ovary, colon, endometrium, brain and eye [35–38].

Late-onset form

The late-onset form has an onset after 40 years [9]. Although some of these patients can be asymptomatic, clinical features such as cataracts, mild myotonia, diabetes and overweight/obesity, are more often in later forms of the disease [10].

3. Genetics in Myotonic Dystrophy Type 1

DM1 is an autosomal dominant inherited disorder, the genetic basis is a cytosine-thymine-guanine (CTG) expansion repeat in the 3'-untranslated region (UTR) of the *DMPK* gene located on the chromosome 19q13.3 [2,39,40]. In the general population, the CTG expansion ranges from 5 to ~35 repeats. Individuals who carry from 36 to 50 CTG repeats show a "pre-mutational" phenotype that is not usually associated with clinical manifestations [10], but may potentially have children affected by the DM1 disease. Symptomatic DM1 patients usually have more than 50 CTGs, and sometimes up to several thousand repeats [41].

The CTG expansion sequence leads to the formation of secondary hairpin-like structures between the guanine-cytosine (GC) groups of the expansion track. These structures are highly slippery and thus, can result into an expansion or a contraction of the repeat during DNA processing events such as DNA replication, recombination, repair and transcription [42,43]. This gives rise to the fact that once the CTG expansion size is more than 36 repeats, it becomes highly unstable [44–47]. However, although contractions can occasionally occur indeed, the CTG repeat track is biased towards expansion [48].

3.1.Anticipation

The instability of the CTG expansion track is highly present in the germline, and tends to expand from one generation to the next [49]. That means that a child of a parent with DM1 may inherit repeat lengths considerably longer than those present in the transmitting parent. This fact results in what is called anticipation, which consists in an increase in symptom severity and a decrease in age of disease onset over successive generations [50]. Thus, in general lines, the CTG expansion increases by more than 200 repeats on average when transmitted from one generation to the next, and the presence of a larger repeat expansion in further generations leads to an earlier onset and a more severe disease [51,52]. The inheritance of the CTG expansion from one generation to another could vary depending on the size of the repeat, the sex [53], and the age of the parent from which the expanded repeat is inherited [54]. The anticipation leading to a congenital DM1 is mostly caused by the inheritance of the expanded mutant *DMPK* allele from the mother [50]. Paternal inheritance of congenital DM1 has been described as well, although is very rare [55,56]. Large expansions may be toxic or negatively selected in sperm compared to oocytes, which could be related to the male infertility that occasionally occurs in DM1 [4]. However, when small CTG expansions are inherited from the father, especially below 100 CTG repeats, the offspring generally shows greater expansions than when the expansion with a similar size is inherited from the mother [45,57,58]. Intergenerational expansions are also affected by the age of the parents, especially when the expanded allele is inherited from the father, meaning that the inherited expansion is higher as higher is the age of the parents [54]. Contractions of the CTG expansion in between generations have also been reported, with a higher estimated prevalence in paternal transmission compared with maternal transmissions (19.5% vs 6.7%)[59].

3.2. Somatic instability

In addition to germinal instability, the CTG expansion is also highly unstable in the soma, at different rates in different cells. This means that the CTG expansion presents size variations within the tissues of an affected individual [60]. Furthermore, the rate of expansion is highly variable among tissues as well [61–65], contributing to the tissue-specific symptoms and the clinical heterogeneity in between patients. In this regard, one study showed that the CTG expansion varied from tissue to tissue after analyzing 22 types of tissue obtained at autopsy from an adult DM1 patient [65]. The study showed that most of the tissues carried longer CTG expansions compared to leukocytes, and only cerebellar tissue carried shorter expansions than those present in leukocytes. Furthermore, different studies have shown that the CTG expansion is larger in skeletal muscle [61,62,66], heart [67], and fibroblasts [63] than in leukocytes. Intracerebellar instability has been reported as well [64], with the smallest expansions present in frontal cortex and thalamus [67].

In addition to reasons of somatic instability such as slippery structures formed through the CTG expansion, DNA processing events and repair and presence of variant repeats, it has been considered that other factors may further affect the somatic instability. However, the mechanisms are still not fully understood. Some studies have shown that somatic instability persists through the lifetime of the patient and is age and size-dependent [68], contributing to the progressive nature of the symptoms [69,70]. Whether the sex could be another modifier, was considered, although a recent study did not found sex-bias in the somatic instability [71].

Altogether, the somatic instability is highly variable within a DM1 patient itself. The CTG size varies in a tissue, between patients' tissues, and depending on the patient age at which the tissues are studied. All these variables affect the establishment of genotype-phenotype relationships.

3.3. Interruptions

Although most DM1 patients carry a pure CTG expansion in the *DMPK* locus, in the last years it has been discovered that around 3-5% of these patients contain interruptions (also known as variant repeats) in the expanded *DMPK* allele [42,72]. The most common variant repeat is cytosine-cytosine-guanine (CCG), although cytosine-

thymine-cytosine (CTC), guanine-guanine-cytosine (GGC), cytosine-guanine-guanine (CGG), and cytosine-adenine-guanine (CAG) have been described as well [42,72–74]. The presence of these variant repeats can be inherited or appear as a *de novo* mutation [75]. Interruptions have been detected in both 5′ and 3′ ends of the CTG expansion, although the 3′ is the most common side where these variants are present [42,72,74,76–78]. CTG variant repeats are known to reduce both germline and somatic instability [72,74,75,79], suggesting a stabilizing effect of the expansion on germline transmission [74,76,77] and also reporting a tendency to contraction of the repeat between generations [42,75]. Variations also appear to be associated to a delayed age of disease onset [42,76,77,79,80]. On the other hand, how the variant repeats affect the phenotype of DM1 patients is still poorly understood, only a few families and some isolated cases have been described in the literature. The phenotypic consequences of these variant repeats vary considerably between studies. Some studies showed that variant repeats are associated with unusual symptoms such as a complex neurological phenotype [72], calf hypertrophy (suggestive of myotonic dystrophy type 2 phenotype) [77], or with a milder severity affectation [75] without muscular dystrophy [42] or without cognitive involvement [78].

4. Technical methods to size the CTG expansion

Determining the length of the CTG expansion in DM1 patients can give us useful information since the number of the CTG repeats is related to the age of disease onset and the severity of the disease [9,70,80]. Furthermore, genetic information can be important for clinical trials.

Although the importance of an accurate length measurement, sizing the CTG expansion is very challenging, not only because of the somatic instability and the immense length of expanded alleles present in a sample but also because of the long CTG tracks and the hairpin secondary structures that are created inside the repetitive GC-rich sequence. The expanded alleles are not always measurable by conventional polymerase chain reaction (PCR), and the presence of these slippery structures hinders the DNA polymerase amplification. Different strategies have been described [47,81,82] and some of them are currently and widely used to assess the CTG

expansion size in patients with DM1, such as long PCR-southern blot and small pool-PCR.

4.1. Long PCR-southern blot (LPCR-SB)

Long PCR-southern blot (LPCR-SB) consists in pre-amplifying the CTG expansion and further southern blotting the amplified genomic DNA. Several long PCR are used, with different primer pairs located in the flanking regions of the CTG expansion and different amplification programs [2,42,47,83]. In the southern blot, a labelled (CAG)n probe binds to the expanded repeats. These approaches typically show expanded alleles as a smear on the blot, due to somatic instability [84], corresponding to different CTG length tracks of different cells. Selecting the sizing point of the diffuse smear on the blot is not easy and very important, since the selected size would be attributed to the patient and sometimes used in genotype-phenotype correlations [85].

4.2. Small pool-PCR (SP-PCR)

The small pool-PCR (SP-PCR), unlike the conventional LPCR-SB, consists in using small amounts of input DNA (180-600 pg) [47]. Thus, few genomic equivalents are separately PCR-amplified and individually detected by southern blot. By using few replicates of PCR reactions per sample, this method provides a detailed quantitative measure of the expansion length variation [70,86], detecting not only a single CTG expansion averaged from thousands of cells, but the distribution of the CTG expansion instability. That means that through this technique the lower boundary can be estimated for the CTG size progenitor allele (the CTG repeat length inherited from the parental generation) [87], the mode allele, corresponding to the most frequent boundary in the blot, and the largest allele, the higher boundary in the blot.

4.3. Heat pulse extension-PCR (HPE-PCR)

Orpana *et al*., developed, in 2013, a modified PCR amplification method to size the CTG expansion in DM1 patients. This method, called heat pulse extension-PCR (HPE-PCR), tries to improve the polymerase extension through long CTG expansions by multiple heat pulses during the expansion step of the PCR. This strategy temporarily destabilizes secondary structures in the template and causes the newly

synthesized strands to dissociate and reanneal continuously. Thus, preventing the strands from losing their position and minimizing the differences in amplification efficiency between short and long alleles [81].

4.4. Tripled-repeat primed PCR (TP-PCR)

The tripled-repeat primed PCR (TP-PCR) technique is not an adequate method to size the CTG expansion, but it is a robust and fast method to identify the expanded allele. Thus, TP-PCR has come into routine as a DM1 diagnostic procedure [88]. It was described by Warner *et al*., introducing a cheaper and faster approach to genetically diagnose DM1 patients compared to southern blot [89]. More recently, Radvansky *et al*., developed an improved bidirectionally labeled TP-PCR in which both forward and reverse directions with two individual fluorescently-labeled flanking primers are used [88]. The technique consists in the use of three primers instead of two. One of the main primers is fluorescently labeled, and the other main primer binds to the CTG repeat and has an extra tail complementary to the third primer. Subsequently, the amplification pattern is visible as a continuous ladder with a 3-base-pair periodicity, due to the CTG triplets. With this method, it can easily be seen if there is an expansion or not. Furthermore, this technique is also useful for the detection of variant repeats, located at the 3' or 5' end. When there is a variant repeat in the CTG expansion, the peak signal drops, showing a gap in between the ladder pattern.

4.5. Other techniques

In recent years, other techniques have been described to assess the CTG expansion in DM1. Lian *et al*. validated a technical approach by combining TP-PCR with melting curve analysis. With this method, DM1 samples can be clearly distinguished from non-DM1 samples by showing higher melting peak temperatures, and CTG expansions up to 180 repeats can be rapidly screened [90,91]. Dandelot *et al*. described a method called flash-small-pool PCR in which they perform the classical SP-PCR using one of the primers labeled with near infrared dye, and denaturing alkaline gel electrophoresis [92]. This strategy reduces experimental steps compared to the classical SP-PCR, and allows to detect up to 1200 CTG repeats in mice. Malbec *et al.*, presented a fast method in which after PCR amplification, the size of the expansion can be determined within 5 minutes [93]. The method is based on advanced microfluidic technologies of DNA concentration, separation and detection

in a single module of a microchip. Leferink *et al*., described FastDM1 ™ *DMPK* sizing kit, based on a TP-PCR which is able to size up to 180 CTG repeats [94]. All these techniques are quite recent and some of them are still pending to be further tested and validated. In the next years, we will see how they develop and whether some of them are widely implemented in the DM1 field.

5. The link between the CTG expansion and the severity of the disease

The general consensus is that the CTG expansion length is related to the severity of the disease, in a way that longer CTG repeat expansions are related to an earlier age of disease onset and a more severe disease [95]. In this regard, and referring back to the subtype classification of DM1, the congenital DM1 patients usually carry larger expansions than the adult DM1 patients (*i.e*., CTG repeat size ≤150 CTGs, ≤1,000 CTGs and >1000 CTGs is common to late-onset, classical adult form and congenital DM1, respectively) [96]. However, there is certain CTG size overlapping between the different DM1 subtypes (**Figure 1**) [5], *i.e.,* congenital cases have been found carrying less than 1000 CTG repeats [40], and adult-onset cases carrying a CTG expansion above 1000 repeats [97], suggesting that other factors should be involved in the determination of the disease severity. Among these factors, over the last years it has been shown that methylation can play a role in DM1 severity, especially in congenital cases, and independently of the CTG expansion length [98]. Moreover, a recent study showed that *DMPK* methylation levels in blood were related to some muscular and respiratory profiles of adult DM1 patients [99].

In the last years, different studies have tried to further analyze the link between the CTG expansion size and the severity in DM1 by not only studying the CTG expansion size distribution among the different DM1 subtypes, but also the relation genotype-phenotype in specific clinical parameters. It is important to identify objective phenotypes with a casual genetic relationship for predicting the progression of the disease and for being applied as an outcome measure in clinical trials. However, the most significant correlation has been reported between the CTG expansion in blood and the age of disease onset, specifically when the CTG expansion is small (<250 CTGs) [82,100]. Whereas the correlations between specific DM1 symptoms

such as neuromuscular involvement [6,101], cardiac involvement [102,103], respiratory affectation [95,104] or cognitive impairment [105] are often poor or

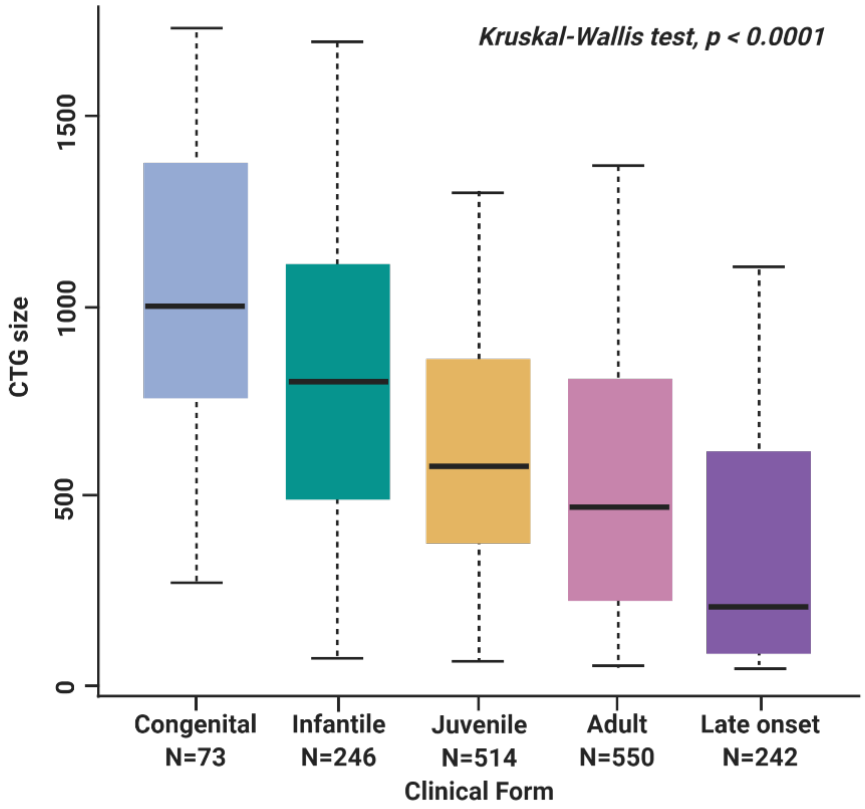


Figure 1. Distribution of the CTG repeat number over the five clinical subtypes of DM1. The distribution of the CTG expansion size differs among the five clinical subtypes. However, there is, in part, an overlap in CTG sizes between the clinical subgroups. Data source from Antonio *et al*. [5]. CTG = Cytosine-Thymine-Guanine; N = number of patients.

absent, providing little conclusive data regarding the CTG repeat number and specific symptoms in DM1.

The analysis of the somatic instability by studying the specific distribution of the CTG expansion size through techniques such as SP-PCR, has been shown to be more accurate and successful in finding genotype-phenotype correlations. Regarding the age of disease onset, studies using SP-PCR technique with a large cohort of DM1 patients showed that the progenitor allele was the major modifier of the age of disease onset, including patients carrying more than 250 CTGs [70]. Likewise, other recent studies have shown that the progenitor allele is one of the major determinants of the

somatic instability [80], and it is strongly correlated with progressive skeletal muscle power and respiratory function [106].

Most of the studies analyzing the relationship between the genotype and the phenotype of patients with DM1, have been focused only on the CTG expansion size that is present in blood. And although it is the most accessible tissue in which the CTG expansion can be easily studied, this fact could be less representative of the status of the other tissues, and be a limitation in finding some correlations especially between tissue-specific symptoms. Moreover, the somatic instability across tissues, within a tissue itself, and the technical difficulties to accurately measure the CTG expansion, are factors that hinder the finding of genotype-phenotype correlations.

6. Molecular mechanisms of Myotonic Dystrophy Type 1

The pathological mechanisms underlying DM1 are very complex. The net that is extended under the CTG expansion repeat is intricate and still not fully understood. The main pathological mechanism of DM1 is based on RNA gain-of-function [107]. Therefore, the toxicity of the repetitive RNA leads to the pathology when the CTG expansion in the DNA is transcribed to cytosine-uracil-guanine (CUG) in the RNA [108,109]. However, although it is well accepted that DM1 is a toxic RNA-triggered disease, some studies have suggested that other mechanisms may simultaneously contribute to the pathological mechanism of the disease, generating consequently the wide spectrum of symptoms and multisystemic manifestations observed in DM1. For instance, it has been shown that the mutant allele of DM1 can be associated to abnormal hypermethylation which is related to congenital DM1 cases and to the maternal transmission, independently of the CTG expansion size [98,99,110,111]. Additionally, it has been seen that the expression of some flanking *DMPK* genes such as six homebox 5 (*SIX5)* or *dystrophia myotonica* WD repeat-containing (*DMWD)* can be affected as well [112–116]. This highlights the importance of the DM1 locus and the effects that can trigger on its vicinity, as a regulatory element that can become dysfunctional and induce transcriptional silencing due to epigenetic changes linked to the enlarged CTG expansion [117] and therefore, contribute to the pathomechanism of DM1 as well. The hypermethylation and reduced expression of *SIX5* in the pathogenesis of DM1, seems to be related with cataracts [118,119], and it

has been hypothesized to be related to male reproductive defects as well [120]. In the case of *DMWD*, a recent study reported that adult *Dmwd*$^{+/-}$ mice shows myofibers reduction [121], suggesting that *DMWD* gene alterations in DM1 patients may affect myofibers.

The molecular mechanisms of DM1 are wide and not all the puzzle pieces are together, yet. Regardless, this thesis is focused on the main molecular mechanism of RNA-gain of function (**Figure 2**) which will be further explained below.

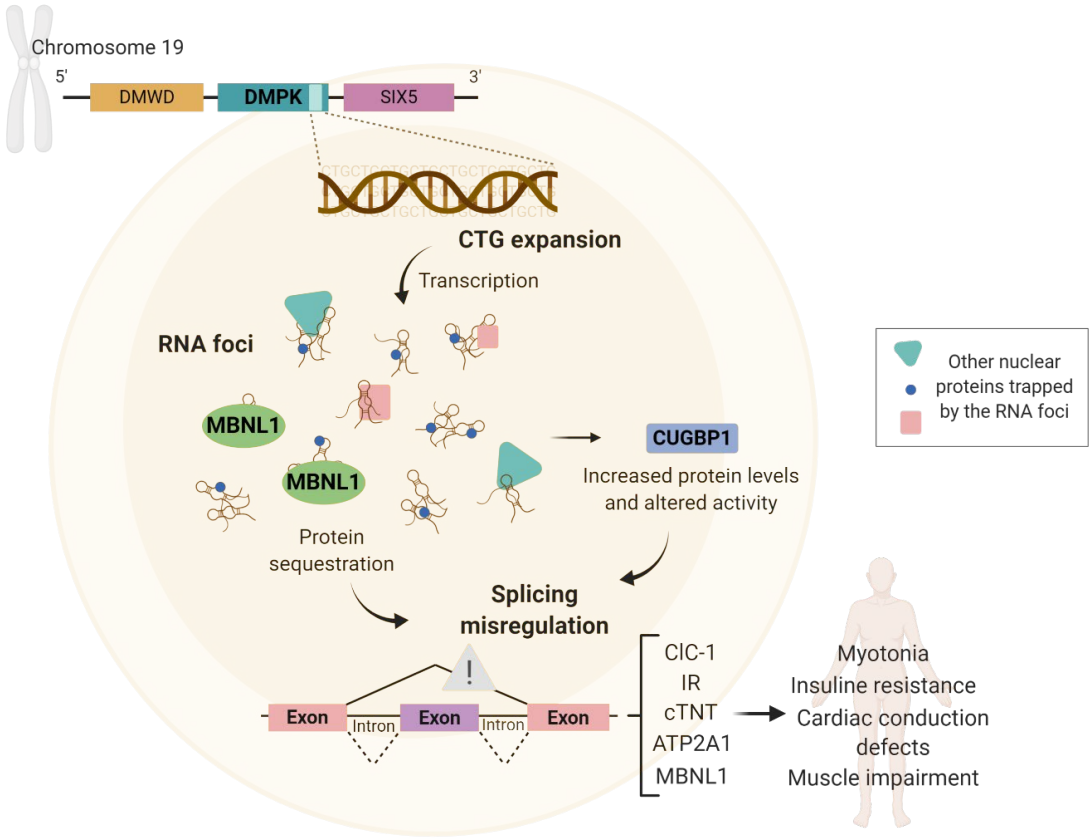


Figure 2. RNA-gain of function mechanism in DM1. In DM1 the presence of the CTG expansion in the 3'-UTR region of the *DMPK* gene, which is flanked by *DMWD* and *SIX5* genes and located in the chromosome 19, causes the nuclear accumulation of toxic RNA aggregates called RNA foci. RNA foci interact with nuclear proteins (turquoise triangle, blue circle and pink square) that can further alter the levels and the activity of proteins such as CUGBP1. RNA foci also sequester nuclear proteins such as MBNL1, which is an important transcription factor involved in the alternative splicing regulation in muscle. The deregulation of both MBNL1 and CUGBP1 causes a disruption of several splicing events affecting ClC-1, IR, cTNT, ATP2A1 or MBNL1 that are related to different clinical manifestations of DM1 patients. DMWD = *Dystrophia Myotonica* WD repeat-containing; DMPK = *Dystrophia Myotonica*-Protein Kinase; SIX5 = Six Homebox 5; CTG = Cytosine-Thymine-Guanine; CUGBP1 = CUG-Binding Protein 1; MBNL1 = Muscleblind-like 1; CLC-1 = Muscle Chloride Channel; IR = Insuline Receptor; cTNT = Cardiac Troponin T; ATP2A1 = Sarcoplasmic/endoplasmic Reticulum Ca^{2+}-ATPase 1.

6.1. From the CTG expansion in the DNA to the RNA foci aggregates

The CTG expansion in the DNA is transcribed to a CUG sequence in the RNA. The mutant transcripts containing the triplet repeats form RNA hairpins are accumulated in the nucleus of the cells as toxic aggregates known as RNA foci (**Figure 2**) [2,39,122]. The relation between the RNA foci number and the CTG repeat size has been poorly explored. One study described that RNA foci differ in number between patients who carry less than 500 CTG repeats and patients who carry more than 1000 CTGs, with an average of 1.18 foci per nucleus (ranging from 0 to 5) and 2.92 foci per nucleus (ranging from 0 to 18), respectively, in muscle tissue [123]. The RNA foci number varies in the different studied samples, in skeletal muscle are found fewer RNA foci than in derived fibroblasts and myoblasts [124–126], and fewer RNA foci are present in fibroblasts and myoblasts compared to myotubes [125,127,128]. RNA foci are known to be dynamic, with liquid-like properties and being able to fuse and divide randomly [129,130]. The location of RNA foci is mainly described in the nucleus of the cells [123–125,130,131]. However, some studies have shown that RNA foci can be detected in cell cytoplasm as well [132,133].

6.1.1. Deregulation of RNA binding factors

The imperfect double-stranded structure of the mutant RNAs causes the deregulation of several RNA binding factors and splicing regulators, including the CUG-binding protein 1 (CUGBP1) and the muscleblind-like (MBNLs) proteins (**Figure 2**) [134–140].

CUGBP1 is one of the first binding proteins that was identified. This protein does not colocalize with the RNA foci, but it has been shown that its expression levels are increased in DM1 due to an inappropriate activation through phosphorus creatine kinase-mediated hyperphosphorylation that is not completely understood yet, and subsequent protein stabilization [141–143]. Functional studies indicate that the increased expression of CUGBP1 is involved in the aberrant regulation of some messenger ribonucleic acids (mRNAs) by binding to U/G-rich motifs in introns adjacent to the regulated splice sites [144–146].

The MBNL protein family (MBNL1, MBNL2 and MBNL3) have been shown to bind to the CUG repeats of the mutant RNA, and colocalize with the nuclear foci

[125,126,136,147]. MBNL protein family belongs to a class of tissue-specific, developmentally programmed regulators of gene expression [148]. They control many aspects of the RNA metabolism, such as alternative splicing and alternative polyadenylation, mRNA localization, translation and stability, and microRNA processing [149]. In this sense, MBNL1 appears to play a predominant role in the DM1 pathogenesis, since it is the most abundant MBNL protein in the adult skeletal muscle, and plays the major role in the alternative splicing regulation of both skeletal and cardiac muscle [150]. Contrary, MBNL2 is relatively abundant in the brain, and it is shown to be involved in many central nervous system features of DM1 [151]. MBNL3 is mainly expressed during the embryonic period and it is generally low in all adult tissues, except for the liver and placenta [152]. The MBNL3 function *in vivo* and its relation to DM1 is not clear yet, but it is suggested to be a contributing factor to progressive skeletal muscle weakness [153,154].

6.1.1.1. Splicing alterations

As a result of the deregulation of several splicing regulators due to the toxic RNA aggregates containing the CUG repeats, more than 400 different proteins suffer alterations in their splicing, changing to a fetal pattern of RNA splicing and playing an important role in the development of DM1 symptoms and pathological DM1 features [109,140,155]. Some of these splicing events include the deregulation of the muscle chloride channel (ClC-1), insulin receptor (IR), cardiac troponin T (cTNT), sarcoplasmic/endoplasmic reticulum Ca^{2+}-ATPase 1 (ATP2A1) and MBNL1 itself (**Figure 2**). The abnormal splicing of ClC-1 increasing the inclusion of exons containing premature stop codons results in down-regulation of ClC-1 mRNA and protein, which leads to reduced chloride conductance in muscle fibers and causes the myotonia sign observed in many DM1 patients [156,157]. Another splicing alteration seen in DM1 is the increased skipping of the IR exon 11 [31,158]. The resulting spliced isoform lacking exon 11 has lower signaling capacity and less insulin sensitivity [159], leading to insulin resistance in DM1 patients. The disruption of the alternative splicing of cTNT is probably related to some cardiac conduction defects of DM1 patients, and it is due to an increased inclusion of cTNT exon 5 [135]. An alternative splicing variant excluding the exon 22 of ATP2A1 is found in skeletal muscle of DM1 patients [160], affecting the muscle development and maturation [161]. MBNL1 has an autoregulatory feedback loop responding to the MBNL content with some spliced

exons [153]. In this sense, it is shown that the inclusion of exon 7 is significantly higher in DM1 [162] and is implicated in increasing MBNL1 affinity to the RNA binding [163,164].

Many studies have shown the direct relation existing between some of these splicing alterations and the deregulation of both MBNL1 and CUGBP1. For instance, it is shown that delivery of MBNL1 restores normal ClC-1 splicing and reverses myotonia [165], and MBNL1 knockdown in cultured cells causes disrupted splicing of both IR and cTNT [138]. On the other hand, other studies showed that CUGBP1 hyperphosphorylation induces aberrant splicing of ClC-1, IR and cTNT [31,135,156]. This fact highlights the idea that many of the splicing alterations may be a result of the combination of the dysregulation of many binding proteins. Furthermore, although some splicing alterations are directly linked to the clinical manifestations such as those affecting CLC-1, IR and cTNT, there is no direct evidence of a cause-effect relationship between some of the DM1 symptoms and the other splicing alterations, meaning that the spliceopathy may not fully explain the multisystemic DM1 spectrum. Additionally, some of these splicing alterations present in DM1, are also seen in other myopathies such as tibial muscular dystrophy [166], whereby the CTG expansion is not present, contributing therefore to the hypothesis that other factors could be involved in the pathological mechanism of DM1.

6.2. DMPK protein

The DMPK protein is a serine/threonine protein kinase which is necessary for the maintenance of skeletal muscle structure and function [167–170]. Its function is widely extended, and still under study. It is known that DMPK modulates muscle ion homeostasis, having a role in muscle contraction and relaxation. DMPK is critical for the modulation of cardiac contractility and the maintenance of proper cardiac conduction activity, probably through the regulation of cellular calcium homeostasis [171,172]. DMPK may also regulate myosin phosphorylation and sarcoplasmic reticulum calcium uptake in myocytes [173], and play a role in myocyte differentiation and survival by regulating the integrity of the nuclear envelope and the expression of muscle-specific genes [174,175].

The *DMPK* gene has 15 exons, and it is described that human *DMPK* produces six major alternatively spliced mRNAs [176]. DMPK isoforms are cell-type, location and

properties specific [170,176]. It is known that most of the isoforms are expressed in many tissues. *In situ* hybridization analysis showed that DMPK mRNA is expressed in a wide range of adult mouse tissues including skeletal muscle, heart, smooth muscle, bone, testis, pituitary, brain, eye, skin, thymus, lung, intestinal epithelium, cartilage and liver, whereas DMPK mRNA is not detected in the ovary, pancreas or kidney [177]. However, DMPK protein is mainly expressed in skeletal and cardiac muscles in humans [178]. Furthermore, there is one isoform that is only found in the human brain, and another isoform of approximately 80-KDalton that is only found in the heart, skeletal muscle and, to a lesser extent, in smooth muscle of humans [179].

The DMPK protein may represent another factor that could contribute to the development of the clinical manifestations in DM1. Several studies have shown that depletion of *DMPK* in mice models results in mild myopathy and cardiac conduction abnormalities similar to the ones observed in DM1 patients [180–182]. Although recently, one study showed that *DMPK* gene depletion did not compromise cardiac or skeletal muscle function in mice [183]. Moreover, mice *DMPK* knockout models did not develop a multisystemic phenotype mimicking DM1, not appearing to be sufficient to manifest the whole clinical picture of DM1.

In DM1, the transcripts from the mutant *DMPK* allele are theoretically retained in the nucleus and therefore they are not efficiently translated [131], thus a partial reduction of DMPK protein should be expected. Regarding DMPK mRNA levels in tissues from patients, although one study reported normal levels of DMPK mRNA in skeletal muscle of adult DM1 patients [184], most of these studies found that the DM1 mutation resulted in a decrease in total DMPK mRNA [185–187]. In accordance with mRNA data, it was found that DM1 subjects presented a low abundance of DMPK protein in heart and skeletal muscle [188]. In skeletal muscles from DM1 patients, DMPK concentration was found to be decreased to about 50% and the protein decrease did not seem to correlate with the CTG repeat length [189].

7. Models of Myotonic Dystrophy Type 1

Since the first description of DM1, considerable knowledge about the disease has been gathered. However, the development of novel relevant disease models

remains of high importance to investigate pathophysiologic mechanisms and to assess new therapeutic approaches. Both animal models and *in vitro* models provide a unique resource for both fundamental and translational research.

Several animal models, including mouse, fly, zebrafish, and worm, have been developed over the past 20 years to investigate DM1 pathological mechanisms, of which the mouse models are the most extensively studied. For example, one of the most relevant mouse models developed in the DM1 field is the *DMPK* knockout mice, that have been generated to test the effect of the reduction of DMPK transcripts on the disease mechanism [180,182]. The toxicity of the expanded transcript has been studied as well with the generation of different models, such as human skeletal actin long repeat length (HSA^{LR}), a transgenic mouse overexpressing untranslated CUG repeats in the skeletal muscle, based on the *HSA* gene, that includes approximately 250 untranslated repeats [190]. Another mouse model carrying larger CTG expansions (300-600 CTGs), called the DM300 mouse, was generated as well [191]. The intergenerational instability in this mouse model has led to the formation of DMSXL mice, carrying between 1000 to 1800 CTG repeats. The DMSXL mouse model exhibits a more severe phenotype and can represent a congenital DM1 model [192]. Furthermore, different mice models have been generated as well to characterize the molecular and physiological effects of MBNL proteins and CUGBP1 [139,146,150,193,194].

Although animal models largely contribute to the knowledge of DM1, any model completely reproduces or recapitulates all aspects of the multisystemic DM1 phenotype. Because DM1 is a multisystemic disease, affecting many tissues and cell types, different cell models are also required to decipher all molecular mechanisms associated with DM1.

In vitro cell models that express exogenous CTG repeats have been used to confirm the direct role of the repeats in the pathologic mechanisms of DM1, for small molecules screenings, therapeutic approaches, or other instigations [135,195,196]. These repeats usually inserted in the 3'UTR of a truncated *DMPK* gene commonly under the control of a cytomegalovirus promoter, are transiently or stably expressed in well-characterized human or murine cell lines, such as HeLa cells, human embryonic kidney cells, or mouse C2 cells [135,138,197]. However, overexpression of large pure repeats in these models is more challenging due to their instability and technical

issues associated with the cloning of long tracts of CTG repeats, restricting substantially their length [198]. Moreover, these models may encounter some limitations associated with the level of CTG overexpression that is not under the control of its own endogenous *DMPK* promoter and the absence of the complete *DMPK* genomic context that could limit their use in specific tissues or molecular mechanism studies.

DM1 patients' derived cell models are of great utility as well, since they can reproduce some of the molecular hallmarks of the disease, such as RNA foci formation, MBNL sequestration, alteration of metabolic pathways and in some cases, alternative splicing dysregulation [136,199–205]. Moreover, they express the whole range of CTG repeats observed in affected individuals within their natural genomic context. In this sense, human primary cells, immortalized human fibroblasts and myoblast, and human pluripotent stem cells have been extensively used [195,201,203,204,206,207]. However, working with DM1 patients' derived cells have also some constraints. The accessibility and availability of the biopsies from patients can be a limitation, as well as the increasing number of divisions or the culture conditions can affect the variability of the results.

8. Therapies for Myotonic Dystrophy type 1

Currently, there is no curative or disease-modifying treatment for DM1. Due to the complex genetic pattern and multifaceted pathophysiology, DM1 patients exhibit extensive variability in the symptomatology. Therefore, an effective, multidisciplinary management monitoring and preventing complications, together with symptomatic treatment (*e.g.*, pain, myotonia, hypersomnolence, *etc*.) is currently critical to help reduce significantly morbidity and mortality in patients with DM1.

Different approaches including gene therapy, genome editing, delivery of exogenously expressed mRNAs, small molecules, and synthetic antisense oligonucleotides (ASOs) are investigated in order to cure DM1 through different interventions. However, RNA toxicity is responsive to the main research therapeutic intervention, and can be approached at several different levels:

(I) Transcriptional silencing. Regarding transcriptional silencing, the reduction of the RNA toxicity has been investigated through the inhibition of RNA polymerase co-factors and through small molecules that bind to CG-rich repeats. Among these molecules, pentamidine, other related antibiotics, and also actinomycin D (a DNA intercalator), can reduce the expression of expanded CUG RNA [208,209].

(II) Post-transcriptional silencing. Various technologies have been used to obtain post-transcriptional silencing of toxic RNA. In fact, it is the main therapeutic strategy in which most of the studies have been focused on. Both using ASOs or small RNAs targeting the CUG repeats [210–212].

(III) Inhibiting interactions between MBNL and toxic RNA. Other studies have focused on inhibiting the interactions between MBNL and toxic RNA, through small molecules or peptides. Common across some of these molecules are aromatic groups that are predicted to intercalate between the U-U (uracil) mismatches or occupy the grooves of CUG-repeat hairpins, displacing MBNL and rescuing partial splicing alterations [213,214].

(IV) Other therapeutic strategies targeting pathways downstream of the RNA toxicity, such as protein kinase C (PKC), glycogen synthase kinase 3 beta (GSK3ß), and adenosine monophosphate activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR), have been investigated as well [143,215,216].

8.1. Antisense oligonucleotides

ASOs are small single or double-stranded pieces of chemically modified nucleotides (10-30 nucleotides) that can target RNA or proteins in a specific manner for therapeutic approaches [217]. There are many modifications available for these compounds that will play a role on defining their properties depending on the therapeutic approach such as specificity to the target binding, stability and pharmacokinetics [218] (**Figure 3**). Natural nucleotides are not suitable for therapeutic application because of their sensitivity to cellular nucleases. Various chemical modifications then, such as phosphorothioate (PS), constrained ethyl nucleotide (2'-

cEt), 2′-O-methoxyethyl (MOE), 2′-O-methyl (OMe), peptide nucleic acid (PNA), phosphorodiamidate morpholino (PMO), and locked nucleic acid (LNA), have been designed progressively over the last years in order to improve the drug properties of ASOs [219,220]. ASOs for therapeutic purposes gained increased importance over the last years, and nowadays there are more than 102 clinical trials listed on ClinicalTrials.gov, eight approved antisense drugs, and several others under consideration and leading many therapeutic research lines/projects [221,222].

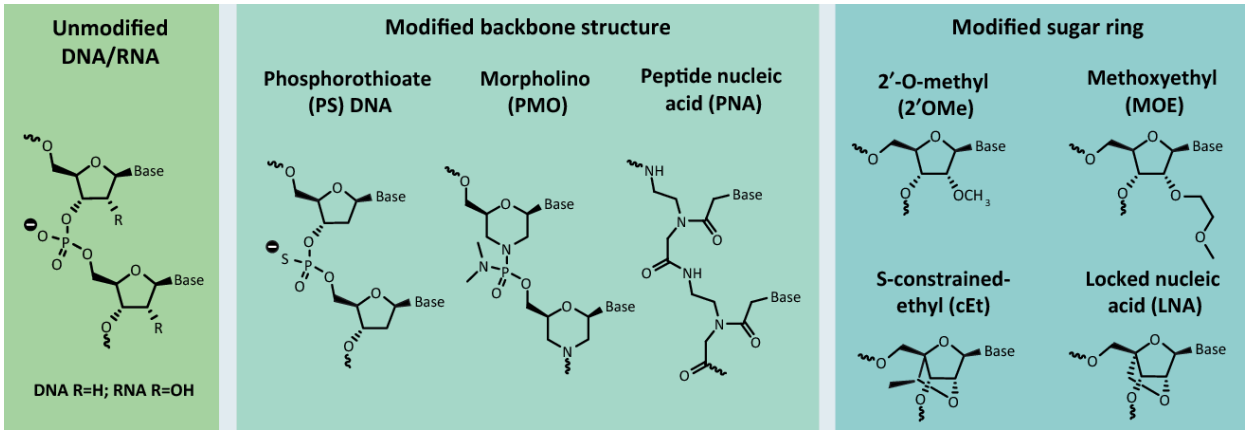


Figure 3. Chemical modifications of ASOs. Unmodified nucleic acids are not suitable for therapeutic approaches since they can be rapidly degraded by nucleases. The chemical modifications help to improve the drug-like properties of ASOs. The phosphate backbone modifications improve the stability, whereas sugar ring modification improve the binding affinity to the target. Figure from Southwell *et al*. [219].

ASOs function via two general mechanisms: steric blocking or targeting enzymatic cleavage by RNase H. Steric blocking ASOs are typically uniformly modified (MOE, OMe, LNA, or PMO) ASOs that bind to the RNA and prevent the binding of factors (proteins or RNAs) without inducing RNA degradation [223]. For example, ASOs specifically designed to block splice sites have been used to redirect the splicing of dystrophin in Duchenne muscular dystrophy and survival of motor neuron 2 in spinal muscular atrophy [224–227]. In contrast, ASOs competent for RNase H mediated degradation are typically designed as ''gapmers'' containing 6–10 central nucleotides with RNase H-competent modifications (PS) and flanked by 3 or 4 nucleotides at the 5′ and 3′ ends that contain modifications that further stabilize the ASOs but are not RNase H competent [223]. On binding to the target RNA, the ''gap'' in the gapmer–RNA duplex allows RNase H-mediated cleavage, enzyme that removes the RNA primer for DNA synthesis on Okazaki fragments during DNA replication. Thus, resulting in specific degradation of the RNA moiety in a gapmer–RNA duplex.

As an example, Mipomersen is a gapmer approved as a drug to reduce the level of low-density lipoprotein cholesterol in patients with familial hypercholesterolemia [228].

The main concerns about ASOs and their therapeutic potentiality, are both delivery and toxicity. ASOs are small molecules and the cellular uptake is primarily an adsorptive endocytosis process, indeed [229]. Some chemical modifications plus the conjugation of peptides or lipid carriers can help on promoting cellular uptake and delivery into cells and *in vivo* models [229–233]. However, despite these advances, to be delivered to the site of action and to produce expected efficacy *in vivo* is still a challenge for ASOs, especially after systemic delivery. Another concern is the toxicity associated to the ASOs, which is mainly related to their backbone chemistry in the attempt to improve stability and pharmacokinetics, but also promoting immune stimulation and non-specific bindings to plasma proteins [234]. Thus, an equilibrium between efficacy, delivery success, and toxicity is important when working with ASOs.

8.1.1. Antisense oligonucleotides as a therapeutic approach for Myotonic Dystrophy Type 1

Among the development of therapeutic approaches in DM1, the antisense therapy has achieved special great success, both *in vitro* and in animal models, to the point to the translation to a human trial. Both steric blocking ASOs and gapmers have been tested in DM1 patient cells and mouse models to target the CUG expanded RNA with high specificity and efficacy, demonstrating great potential for future DM1 treatment [210,235–237] (see **Figure 4** for steric blocking and RNase H activation mechanisms comparison). However, the most attractive approach to eliminate RNA toxicity and the strategy that is further used in clinical development employs RNase H1 activity to directly degrade the toxic DMPK transcript. To note, among all chemical modifications, only the PS backbone modification is compatible with endogenous RNase H activity [238].

Several studies have demonstrated the high efficacy and the potency of using RNase H-active ASOs in targeting the CUG expanded RNA in both DM1 cell culture and mouse models [196,210,237]. Wheeler *et al*., showed that in the DM1 mouse model HSA^{LR} a MOE gapmer ASO caused marked reduction of CUG expanded RNA in skeletal muscle, accompanied by the release of MBNL protein from foci, correction

of splicing errors, elimination of myotonia, and improvement of muscle architecture [210]. These findings spurred efforts to develop an optimized *DMPK*-targeting ASO and later on, they shown a reduction of DMPK RNA in the muscle of mice and non-human primates (Cynomolgus Monkey) [237]. Furthermore, a dose-finding safety trial was conducted in adult DM1 patients by ISIS pharmaceuticals using a gapmer with both 2'MOE and 2'cEt modifications (IONIS-DMPK$_{Rx}$) (NTC02312011). However, although the drug was safe and well tolerated, and some of the splicing biomarkers went in the right direction, the overall concentration of the ASO achieved in muscle was considered not high enough to likely have a clinical impact. Thus, the trial was halted. ISIS pharmaceuticals is currently working on modifying the oligonucleotide to improve delivery efficiency.

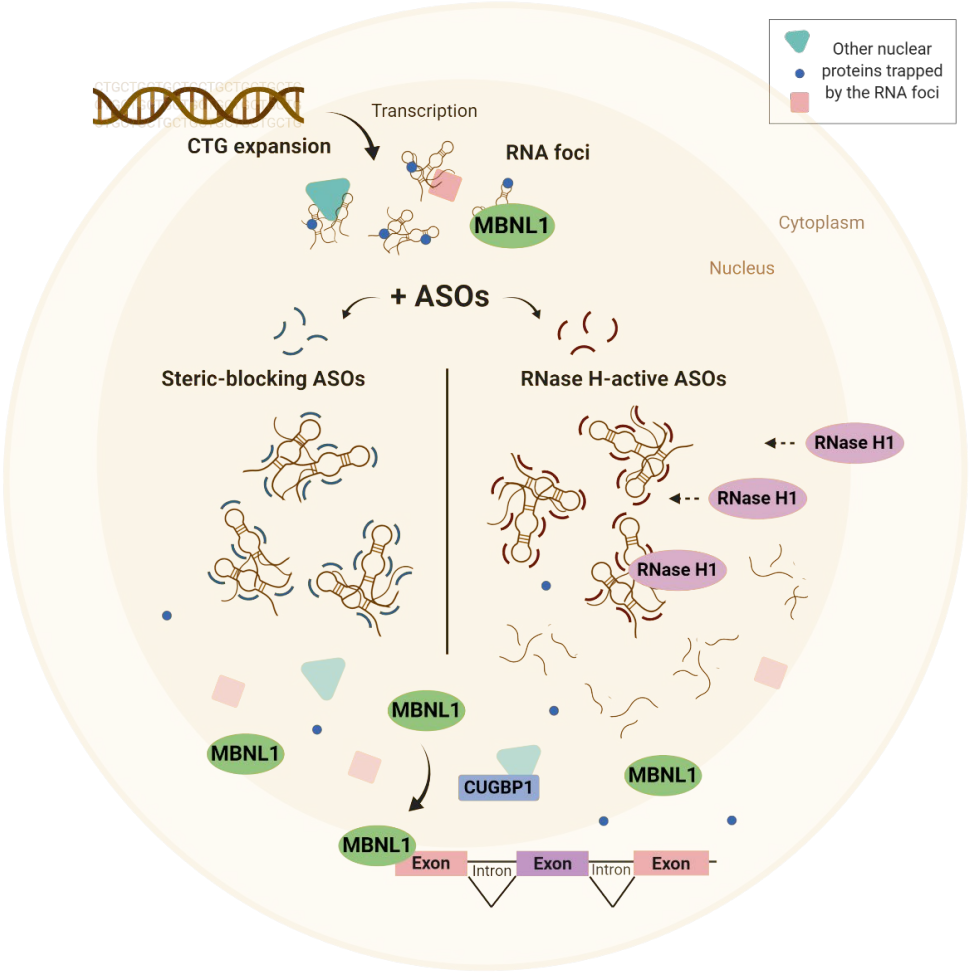


Figure 4. Steric blocking and RNase H activation ASOs mechanisms. Steric blocking and RNAse-H activation are the two ASOs mechanisms used in DM1. With the steric blocking mechanism, the ASOs (in blue) block the access to nuclear proteins such as MBNL1 and prevent the deregulation of missplicing events. With the RNase H activation mechanism, the most commonly studied, ASOs (in red) recruit the enzyme RNase H which degrades the toxic RNA. CTG = Cytosine-Thymine-Guanine; MBNL1 = Muscleblind-like 1; CUGBP1 = CUG-Binding Protein 1.

8.1.1.1. Bridged nucleic acids

The rapid progress in nucleic acid research and the attempt to improve the properties of ASOs, led to the progressive design of compounds that included more drastic modifications and structural changes. One example of the last generations of ASOs is LNA. LNA compounds were first introduced in the late 1990s, and are bicyclic nucleotide analogs in which the furanose ring is modified by the introduction of a methylene group linking the 2′-oxygen and the 4′-carbon (2′-*O*,4′-methylene-β-d-ribofuranosyl nucleotides) [239–242] (**Figure 5**). They are characterized by reduced flexibility and a locked *N*-type conformation which favors the formation of stable duplexes with DNA or RNA [239]. Comparison of LNA and MOE gapmers (which is the AOs that was under DM1 clinical trial) indicates that LNA gapmers are 5–10 fold more potent, eliciting initial interest as a more potent alternative to MOE gapmers [243,244]. However, while the increased binding capacity is advantageous, the high affinity of LNA-containing oligonucleotides can also result in toxic effects due to unspecific off-target binding. Although recent studies showed that LNA-containing oligomers were innocuous in primates [245] and relatively safe in humans [246–248], other studies showed hepatoxicity in both mice and cell models [244,249,250]. Altogether, this fact stimulated the search for similar LNA compounds, improving their properties. In this sense, 2′,4′- bridged nucleic acid (BNA^{NC}) [251–253] has been recently introduced. These LNA analogs can include different substitutions at the N atom, of which a methyl group is the most commonly used to date (**Figure 5**).

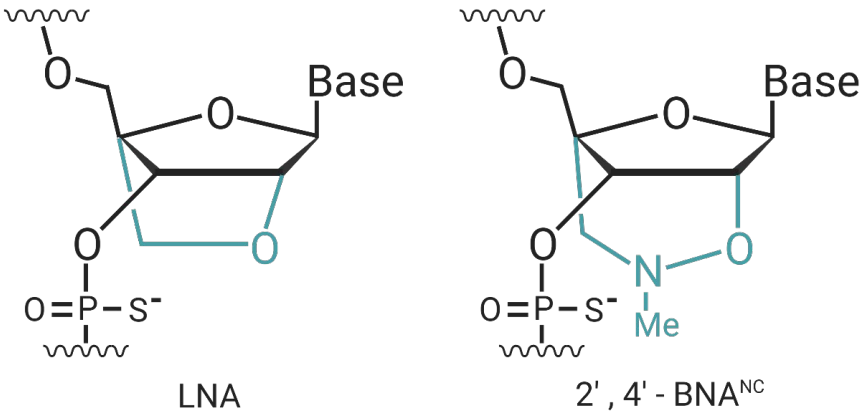
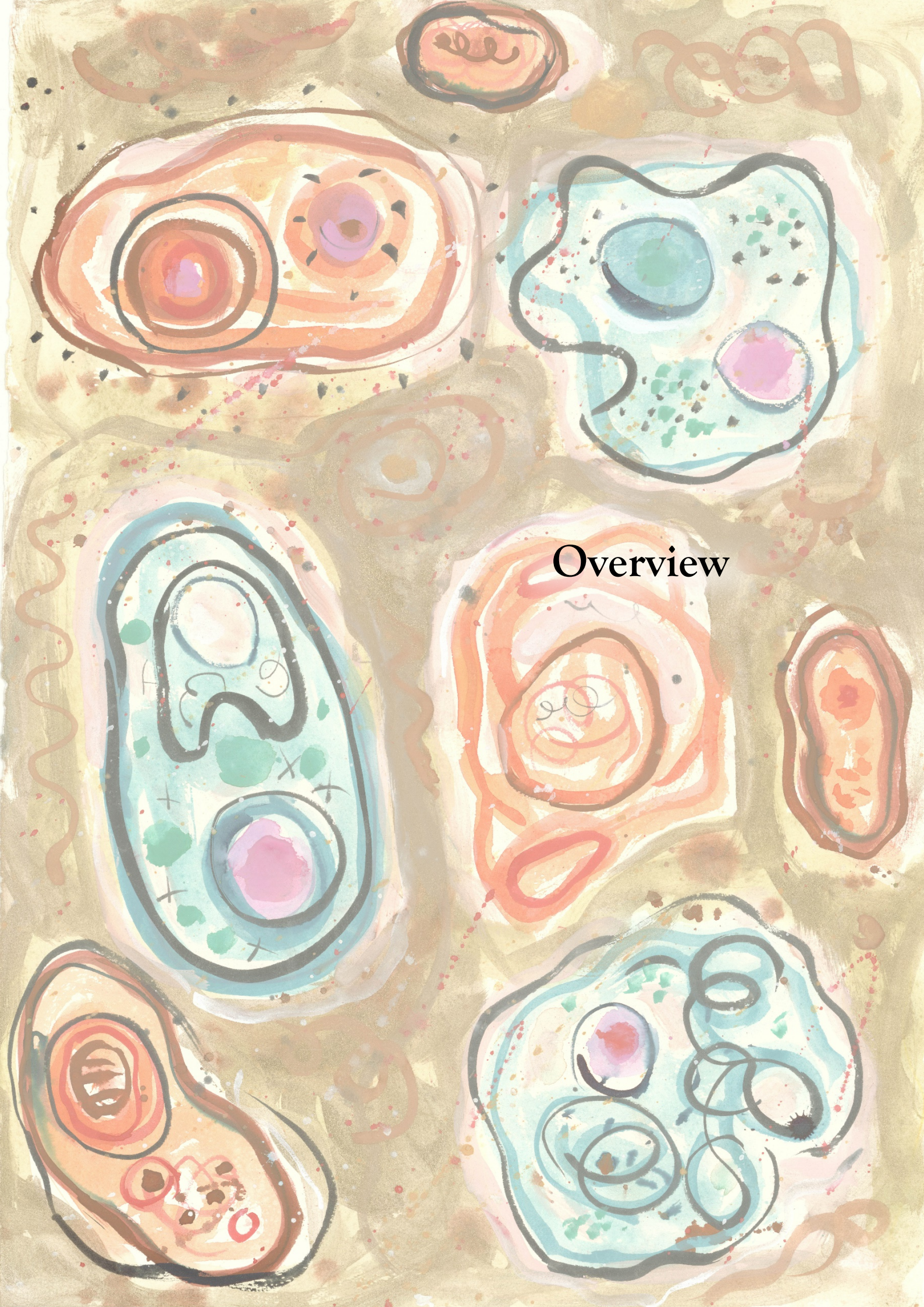
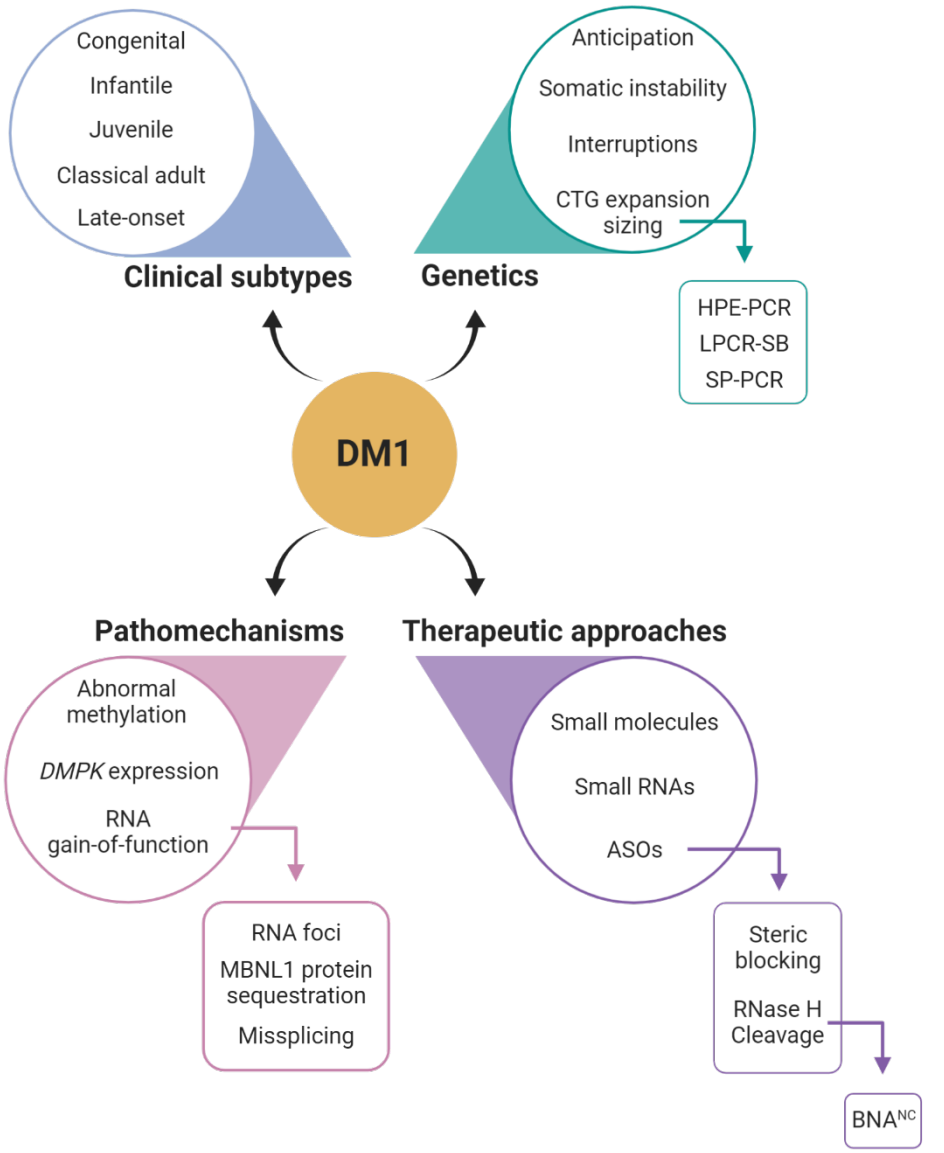


Figure 5. LNA and 2′,4′ – BNA^{NC} oligonucleotide modifications. LNA and BNA compounds have a bridging structure (marked in blue) by which the degree of freedom in conformation is restricted to increase the binding affinity to target the toxic RNA. LNA = locked nucleic acid; 2′,4′ – BNA^{NC} = 2′,4′- bridged nucleic acid.

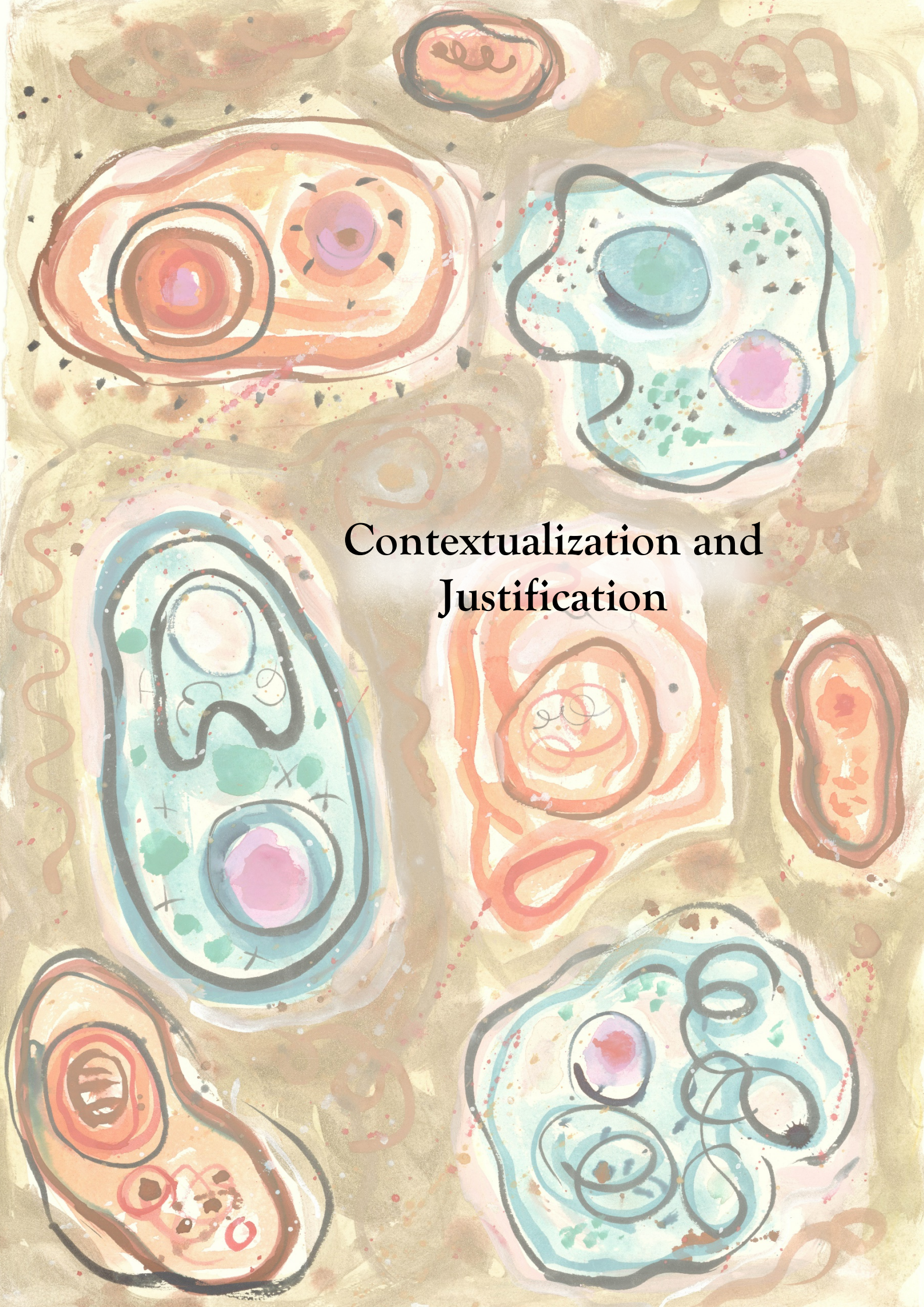
BNA^{NC} are more stable than LNA ASOs and have been shown to be well tolerated in mice [252,254]. Furthermore, a study showed the potential of BNA^{NC} gapmers (2′4′-BNA^{NC}[NMe]) as a promising alternative chemistry for therapeutic development in DM1. In the mentioned study, the authors show that BNA^{NC} gapmers have comparable potency to LNA gapmers, display a potentially lower propensity to induce caspase activity, and functionally rescue characteristic DM1 defects in transfected COSM6 cell line including ~300 CUG repeats [255]. Thus, BNA^{NC} gapmers warrant further study as a promising RNA targeting therapy in DM1.



Overview



General overview of the thesis content. DM1 is a complex disease with a wide variability in the clinical phenotype that can be divided into five main subtypes: congenital DM1, infantile form, juvenile form, classical adult form and late-onset form. The genetics of DM1 is also challenging, and embraces different concepts: anticipation, somatic instability and interruptions (or variant repeats). Moreover, no gold standard exists to size the CTG expansion, and different approaches are currently used in DM1 laboratories, including HPE-PCR, LPCR-SB and SP-PCR. The main pathomechanism in DM1 is the RNA gain-of-function, where the presence of RNA foci and the subsequent MBNL1 sequestration leads to missplicing. However, the *DMPK* expression depletion may contribute to the DM1 clinical manifestations as well. Moreover, over the last few years, it has become clear that the DM1 pathogenicity is far more complex and various additional molecular pathomechanisms such as abnormal methylation are under investigation. Finally, DM1 has no cure yet, but many research studies have tested small molecules, RNAs and ASOs as a possible treatment for DM1. However, ASOs with RNase H cleavage is the further studied approach to systematically treat patients with DM1. Although the delivery and the toxicity of these compounds pose challenges, new improvements in their chemical properties open the possibilities to new promising molecules such as BNA^{NC} compounds.



Contextualization and Justification

Five years ago, in 2015, Dr. Gisela Nogales Gadea started a new research group together with the Neuromuscular Pathology Unit of the *Hospital Universitari Gemans Trias i Pujol*. The research laboratory group was settled in the *Mar* building of the *Health Research Institute Germans Trias i Pujol* of the *Campus Can Ruti*, in Badalona, Barcelona. Although the group was fully equipped with clinical experts, Gisela was alone in the laboratory. Thus, she looked for somebody to join the group and help her to start a research project on DM1. This person turned out to be Alfonsina Ballester López, the author of this thesis, who was very lucky to end up in that newborn but promising research group. Over the following months, the translational research group composed of both clinical and basic researchers was named the *Neuromuscular and Neuropediatric research group*. The group began to grow, attending to conferences, reporting the first results, making collaborations and most importantly, incorporating new members to the team who made it possible to make huge advances in the field. Five years later, we have investigated the complexity of DM1 from different aspects, concluding into a global research approach that is reflected in the thesis that is in your hands today.

We divided the present thesis into five chapters, corresponding to different unknowns related to DM1, going from the technical challenge of sizing the CTG expansion of the patients, to the analysis of a potential treatment for the disease. Our studies include patients with juvenile, adult and late-onset DM1.

The first problem we faced from the very beginning was the challenge of sizing the CTG expansion in patients with DM1. On one hand, the somatic instability and on the other hand, the long CTG tracks hinder the amplification and the accuracy of the repeat measurement. This has led to the use of a number of different strategies to size the CTG expansion in these patients. However, it remains to be determined whether the different methodologies yield similar results when sizing the CTG expansion. (**Chapter I**)

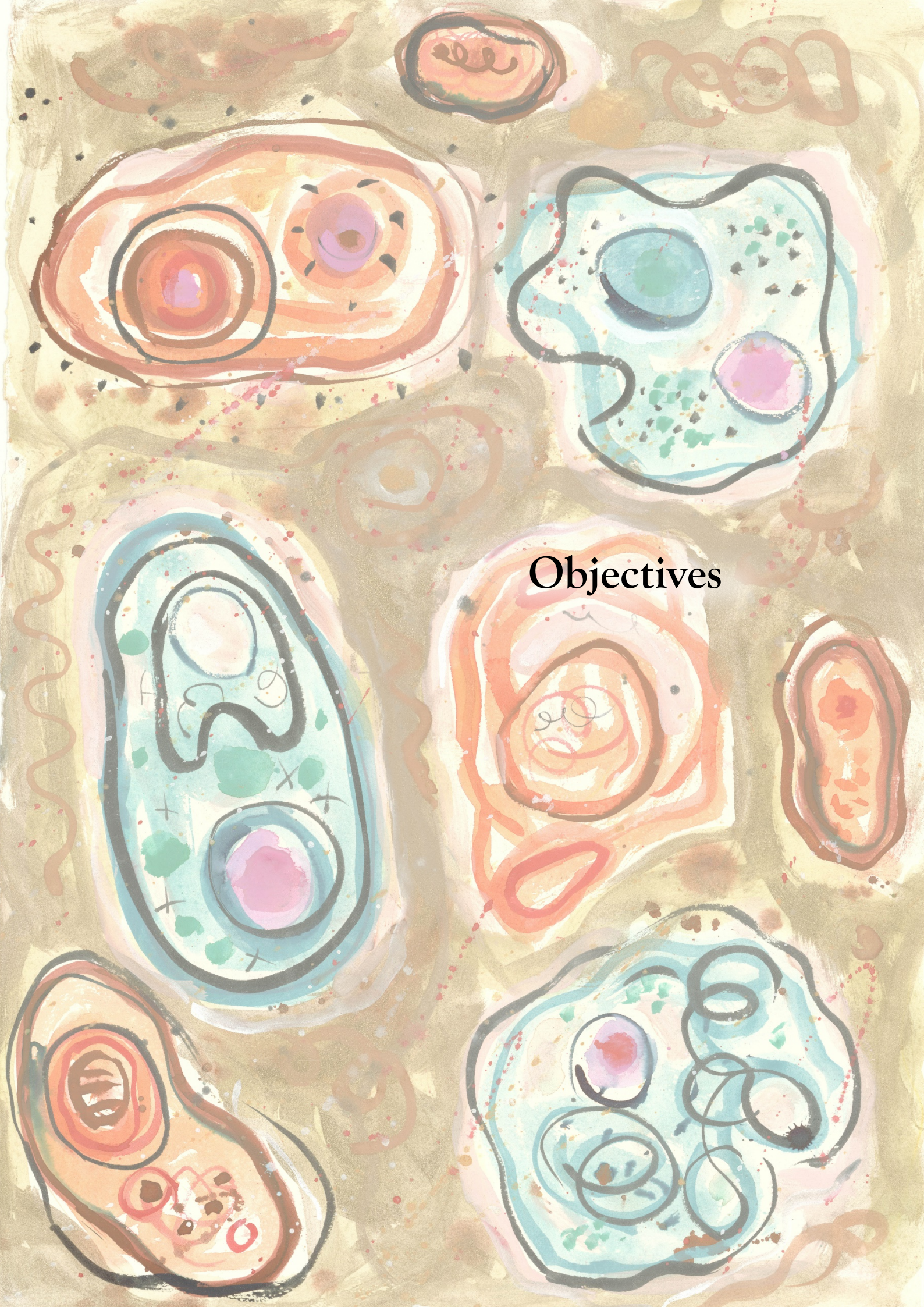
The study of the CTG expansion size differences between and within tissues has been studied so far using techniques with limited sensitivity. However, the analysis of the CTG expansion between tissues using the SP-PCR technique, which allows the study of the allele distribution, would provide a better characterization of the somatic instability not only between tissues but also within tissues, and thus, a better

understanding of genetic associations with the clinical manifestations in DM1 patients. (**Chapter II**)

The presence of variant repeats (interruptions) in the CTG expansion is known to affect the expansion's inheritance dynamics and also the phenotype traits of DM1 patients. However, only few families and some isolated cases have been described, and the phenotypic consequences of variant repeats remain poorly understood and vary between studies. There is a need to determine the phenotypes associated with the subset of DM1 patients presenting variant repeats. This information is required for patient management, genetic counseling and future clinical trials. (**Chapter III**)

Regarding the complexity in the molecular mechanisms that underlie DM1, the RNA-gain of function is considered one of the most relevant, and it has been widely studied. However, there is incomplete information. Most studies have relied on two-dimensional imaging at a single cell level. Yet, this 'classic' approach does not allow a full overview of the cell and its molecular components, hindering the recognition of the full cascade of molecular events and the relationship between them, affecting the association to the phenotype manifestation of DM1 as well. (**Chapter IV**)

DM1 is a multisystemic disease without a cure. ASOs are potential molecules to cure DM1. However, due to the multisystemic nature of the disease, it is uncertain how the different tissues are going to be affected by the therapy. Therefore, it is important to study different cell lines derived from DM1 patients to analyze both the efficacy and toxicity of the potential treatment. (**Chapter V**)



Objectives

Chapter I

- Determine whether three different techniques used to size the CTG expansion (HPE-PCR, LPCR-SB and SP-PCR) yield similar results when sizing the CTG expansion in blood samples of 15 DM1 patients.

Chapter II

- Analyze the CTG expansion size through SP-PCR in three different tissues (blood, muscle and skin) obtained simultaneously from eight DM1 patients, to understand the tissue´s somatic instability and determine associations with DM1 patients' phenotype.

Chapter III

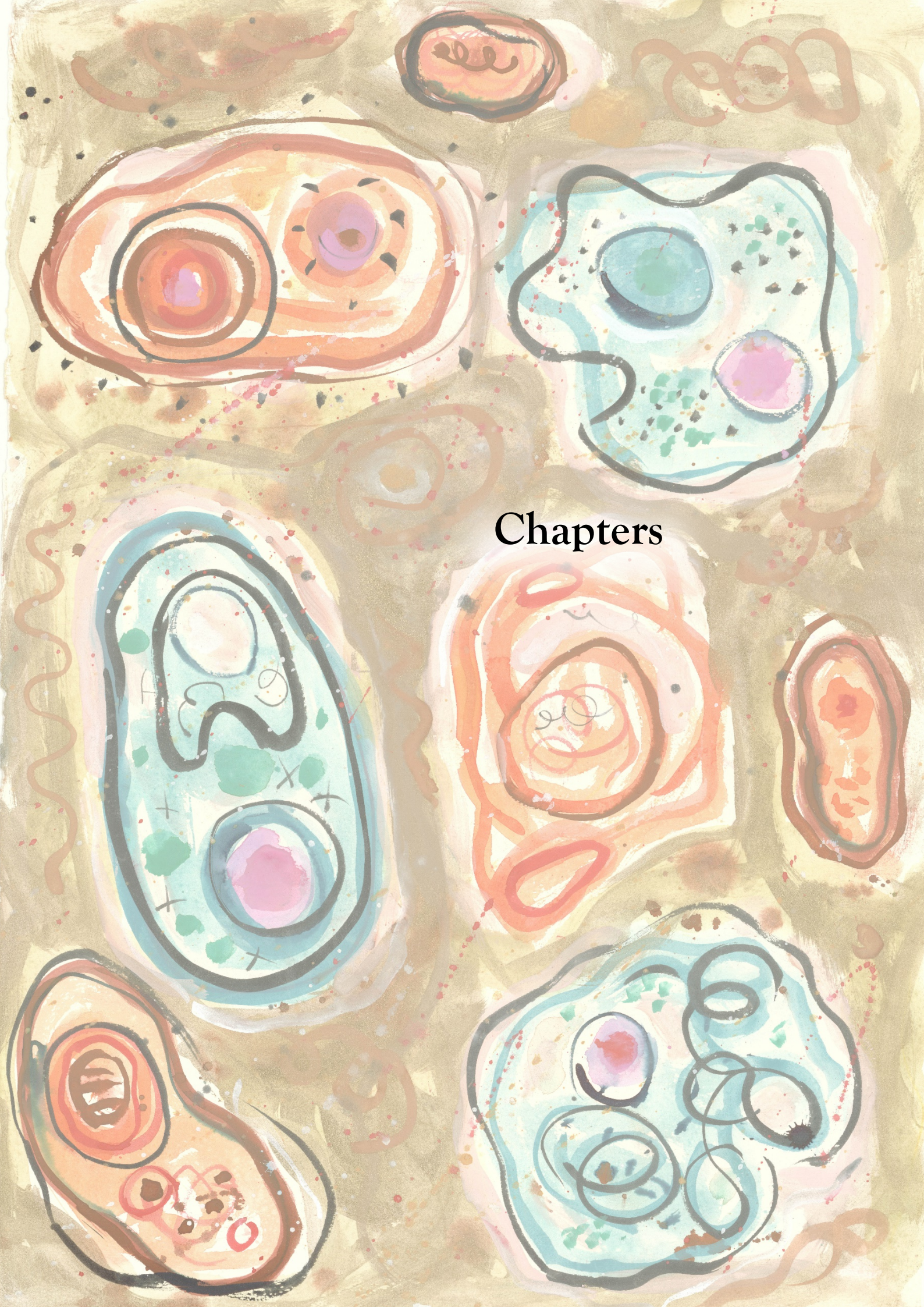
- Identify variant repeats in a cohort of 49 Spanish DM1 patients and analyze if there are special traits in their phenotype, which differentiate them form DM1 patients who carry a pure CTG expansion.

Chapter IV

- Study the CTG repeat size, the RNA foci, the MBNL1 sequestration, the *DMPK* gene expression levels and the splicing alteration in DM1 myoblast derived from six DM1 patients, by means of three-dimensional (3D) imaging and study the relationship between these molecular findings and the associations with the patients' phenotype.

Chapter V

- Evaluate BNA^{NC} treatment toxicity and efficacy in three different patient derived cells (fibroblast, lymphoblasts and myoblasts) of eight DM1 patients.



Chapters

22 de Octubre de 2020

A quien corresponda,

En la presente tesis de Alfonsina Ballester López están incluidos tres artículos publicados, y un cuarto en revisión, que esperamos que en las próximas semanas sea aceptado para su publicación.

Entre los tres artículos aceptados se encuentran:

1. ***A DM1 family with interruptions associated with atypical symptoms and late onset but not with a milder phenotype***. Se publicó en 2019, en la revista internacional *Human Mutation*, que tiene un factor de impacto de 4,370. En este trabajo Alfonsina Ballester es primera autora firmante. Ha hecho la mayoría de los experimentos, ha analizado sus resultados, y ha participado en la escritura y revisado el contenido final del manuscrito.

2. ***The Need for Establishing a Universal CTG Sizing Method in Myotonic Dystrophy Type 1.*** Que se publicó en la revista internacional *Genes* y tiene un factor de impacto en 2019 de 3.331. En este trabajo Alfonsina Ballester es primera autora firmante. Ha hecho la mayoría de los experimentos, ha analizado sus resultados, y ha escrito el manuscrito y revisado el contenido final del manuscrito.

3. ***Three-dimensional imaging in myotonic dystrophy type 1: Linking molecular alterations with disease phenotype*.** Que se publicó en la revista internacional *Neurology Genetics* y tiene un factor de impacto en 2019 de 3.65. En este trabajo Alfonsina Ballester es primera autora firmante. Ha hecho la mayoría de los experimentos, ha analizado sus resultados, y ha escrito el manuscrito y revisado el contenido final del manuscrito.

Todos los trabajos anteriormente citados no han sido utilizados para la realización de ninguna otra tesis anteriormente

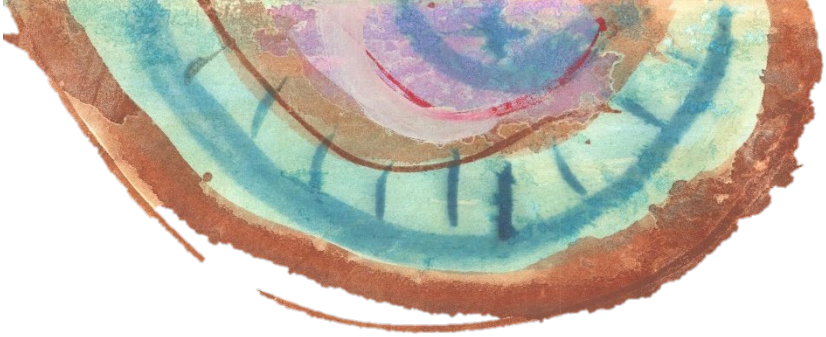
Atentamente,

Gisela Nogales Gadea

(Directora de tesis)

Josep Saura Martí

(Tutor de tesis)



Chapter I

The Need for Establishing a Universal CTG Sizing Method in Myotonic Dystrophy Type 1

Alfonsina Ballester-Lopez[1,2], Ian Linares-Pardo[1], Emma Koehorst[1], Judit Núñez-Manchón[1], Guillem Pintos-Morell[1,2,3], Jaume Coll-Cantí[1,2,4], Miriam Almendrote[1,4], Giuseppe Lucente[1,4], Andrea Arbex[1,4,5], Jonathan J. Magaña[6], Nadia M. Murillo-Melo[6], Alejandro Lucia[7], Darren G Monckton[8], Sarah A Cumming[8], Alba Ramos Fransi[1,4], Alicia Martínez-Piñeiro[1,4], Gisela Nogales-Gadea[1,2]

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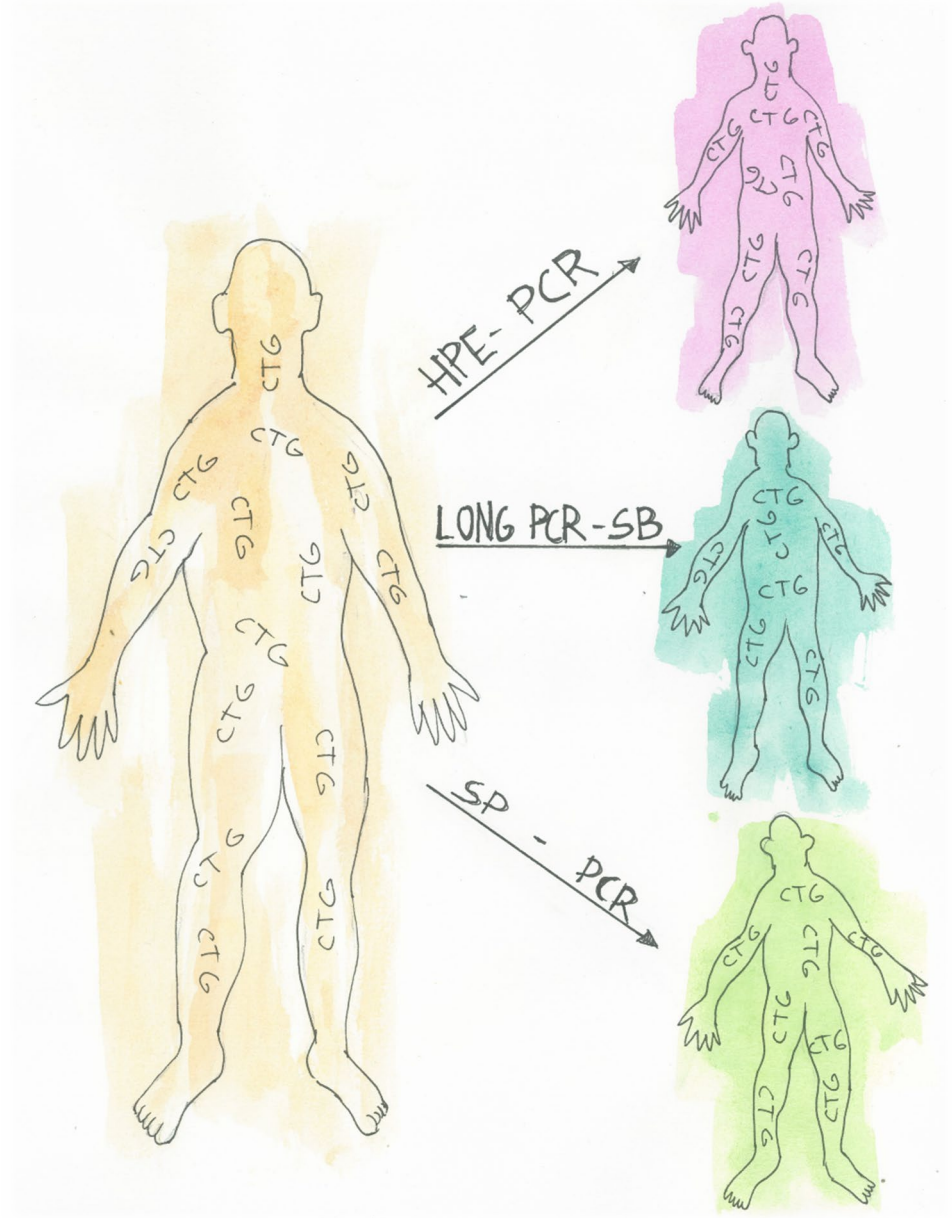
Genes (Basel). 2020 Jul 7 [cited 2020 Jul 8];11(7):757.

Available from: https://www.mdpi.com/2073-4425/11/7/757



genes

MDPI

Brief Report

The Need for Establishing a Universal CTG Sizing Method in Myotonic Dystrophy Type 1

Alfonsina Ballester-Lopez [1,2], Ian Linares-Pardo [1], Emma Koehorst [1], Judit Núñez-Manchón [1], Guillem Pintos-Morell [1,2,3], Jaume Coll-Cantí [1,2,4], Miriam Almendrote [1,4], Giuseppe Lucente [1,4], Andrea Arbex [1,4,5], Jonathan J. Magaña [6], Nadia M. Murillo-Melo [6], Alejandro Lucia [7], Darren G. Monckton [8], Sarah A. Cumming [8], Alba Ramos-Fransi [1,4], Alicia Martínez-Piñeiro [1,4,†] and Gisela Nogales-Gadea [1,2,*,†]

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Abstract: The number of cytosine-thymine-guanine (CTG) repeats ('CTG expansion size') in the 3'untranslated region (UTR) region of the *dystrophia myotonica*-protein kinase (*DMPK*) gene is a hallmark of myotonic dystrophy type 1 (DM1), which has been related to age of disease onset and clinical severity. However, accurate determination of CTG expansion size is challenging due to its characteristic instability. We compared five different approaches (heat pulse extension polymerase chain reaction [PCR], long PCR-Southern blot [with three different primers sets—1, 2 and 3] and small pool [SP]-PCR) to estimate CTG expansion size in the progenitor allele as well as the most abundant CTG expansion size, in 15 patients with DM1. Our results indicated variability between the methods (although we found no overall differences between long PCR 1 and 2 and SP-PCR, respectively). While keeping in mind the limited sample size of our patient cohort, SP-PCR appeared as the most suitable technique, with an inverse significant correlation found between CTG expansion size of the progenitor allele, as determined by this method, and age of disease onset ($r = -0.734$, $p = 0.016$). Yet, in light of the variability of the results obtained with the different methods, we propose that an international agreement is needed to determine which is the most suitable method for assessing CTG expansion size in DM1.

Keywords: CTG expansion size; myotonic dystrophy type 1; long PCR; small pool-PCR; age of disease onset

1. Introduction

Myotonic dystrophy type 1 (DM1) is a multisystemic disorder with an autosomal dominant inheritance pattern. DM1 is caused by unstable expansion of CTG repeats in the 3′ untranslated (UTR) region of the *dystrophia myotonica*-protein kinase (*DMPK*) gene [1]. Non-affected individuals usually have 5 to 35 CTG repeats, with carriage of 35 to 49 repeats, leading to a "pre-mutational" phenotype that is not usually associated with clinical manifestations. In turn, patients with DM1 typically have more than 50 CTG repeats in the blood, and sometimes up to several thousands [2]. Furthermore, because CTG expansion is highly unstable and prone to increase in these patients, an eventual decrease (or 'contraction') in the number of CTG repeats is typically biased toward further expansion in the context of DNA replication, recombination, transcription and/or repair [3–7]. Consequently, CTG expansion shows a characteristic somatic mosaicism [8].

Determination of the actual number of CTG repeats is complicated by the immense length of the expanded alleles as well as by the highly stable secondary structures that are created inside the repetitive guanine-cytocine (GC)-rich sequence. In addition, it is possible to underestimate rare mutant molecules in both germline and somatic cells. Accurate assessment of CTG expansion size is important in patients with DM1. Indeed, the number of CTG repeats can be inversely and directly related with age of disease onset and clinical severity, respectively [9,10]. Although different approaches have been described to assess CTG expansion size in patients with DM1 [5,11–15], some methodological issues remain to be solved, mainly related to the inherent repeat instability and technical difficulties when amplifying long CTG fragments.

It was therefore the purpose of this study to compare the number of CTG repeats ('expansion size') of the progenitor allele and the mode allele between five different assays using three different methods: (i) heat pulse extension (HPE)-polymerase chain reaction (PCR); (ii) long PCR (LPCR)1-Southern blot (SB); (iii) LPCR2-SB; (iv) LPCR 3-SB; and (v) small pool (SP)-PCR. The rationale behind this work was to determine whether the different methodologies that are currently available yield comparable results, so that it is necessary—or not—to come to a consensus as to which methodology should be used.

2. Materials and Methods

2.1. DNA Extraction and Subjects

This study was approved by the Ethics Committee of the University Hospital Germans Trias i Pujol (Badalona, Spain) and was performed in agreement with the Declaration of Helsinki for Human Research in 1975. All subjects signed a written informed consent to participate in the study. Total genomic DNA was obtained from peripheral blood in 15 patients with DM1 and 10 controls, as previously described [16]. Diagnosis of DM1 was confirmed by triplet primed-PCR, as reported elsewhere [17]. In controls, CTG number in both *DMPK* alleles was assessed by Sanger sequencing.

2.2. Heat Pulse Extension-PCR

First, we amplified the genomic DNA from patients and controls using HPE-PCR, as described elsewhere [13]. Unlike conventional PCR, in which the extension step is performed at a constant temperature, HPE-PCR includes multiple heat pulses in the extension step. Heat pulses temporarily destabilize the secondary structures formed in the long GC-rich repetitive sequence, thereby improving the extension efficiency and the amplification of the long expansions. The technique was carried out using the same PCR conditions, reagents, the equivalent taq polymerase (Phusion High-Fidelity DNA Polymerase, Thermo Fisher Scientific; Waltham, MA, USA) and the same thermocycler (GeneAmp 9700

thermal cycler, Applied Biosystems, Foster City, CA, USA) as the Orpana et al. study [13]. The sizing of the CTG expansion was performed in a 1% agarose gel using the molecular ladder NZYDNA Ladder III (NZYTech, Lisboa, Portugal).

2.3. Long PCR-Southern Blot

The DNA samples (100 ng per reaction) were amplified with three different primer sets in a LPCR: (i) DM102 and DM101 (LPCR1); (ii) MDY1D and SOMY4R (LPCR2); (iii) MDY1D and DM1rev (LPCR3). We used the LongAmp® Taq PCR Kit (New England BioLabs, Ipswich, MA, USA) and the GeneAmp 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The set conditions for each of the three LPCR methods were as follows: initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, and annealing–extension at 65 °C for 7 min. Final extension was performed at 65 °C for 10 min. Subsequently, LPCR products were analyzed using SB. In brief, all LPCR products were electrophoresed in a 0.8% agarose gel at 80V for 90 min, and transferred to a nylon membrane (Roche; Basel, Switzerland) after gel washing with an acid solution (250 mM HCl) for 15 min, a basic solution (0.5M NaOH) for 30 min, and a neutralizing solution (0.5 M Tris-HCl, pH = 7.5, 1.5 M NaCl) for 30 min. DNA was fixed to the membrane by incubation for 1 h 15 min at 65 °C. We used a concentration of 10 pmol/mL DIG-labeled LNA probe (5′-gcAgCagcAgCagCagcAgca-3′, where capital letters indicate LNA nucleotides) to hybridize the membrane for 3 h at 70 °C. Expansion size was determined by chemiluminescence yielded by the binding of alkaline phosphatase-conjugated to anti-DIG antibody and CDP-Star substrate, according to the manufacturer's instructions (Roche).

2.4. Small Pool-PCR

As opposed to the conventional PCR-Southern Blot, the SP-PCR technique uses small amounts of input DNA, allowing the study of single genomic equivalents, which are represented as single bands in the gel. SP-PCR was carried out using 300 pg of DNA in four replicates per sample, in order to study a representative repeat length distribution of the sample. We used the flanking primers DM-C and DM-DR as previously described [5,18], using a custom PCR Master Mix (Thermo Fisher Scientific; Waltham, MA, USA) supplemented with 69 mM 2-mercaptoethanol, and Taq polymerase Thermus aquaticus (Sigma-Aldrich; Gillingham, UK) at 1 unit per 10 µL. All reactions were supplemented with 5% DMSO and the annealing temperature was 63.5 °C. DNA fragments were resolved by electrophoresis on a 1% agarose gel, SB was hybridized using GE Healthcare Nylon Hybond N Membrane (Thermo Fisher Scientific; Waltham, MA, USA) as described [5,18], and autoradiographic images were scanned.

For LPCR-SB and SP-PCR, the CTG size of the progenitor allele and the mode allele (i.e., yielding the most intense band signal) of each patient were estimated by comparison against the molecular weight ladder, using GelAnalyzer 19.1 software. The length of the flanking CTG region of each PCR was subtracted for all the estimated CTG lengths.

2.5. Statistical Analysis

After checking that the data followed a normal distribution with the Kolmogorov–Smirnov test, we used a repeated-measures one-way analysis of variance (ANOVA) test for performing within-subject comparisons of the mean values of CTG expansion size of the progenitor allele and of the mode allele, respectively, obtained with the different methods. We also calculated the Pearson correlation between (i) the results yielded with the different methods, and (ii) the results obtained with each method and the age of disease onset, the Muscular Impairment Rating scale (MIRS), and the modified Rankin Scale (mRS), respectively, in the 15 DM1 patients. All statistical analyses were conducted using a statistical software package (SPSS 23), setting the significance level at $\alpha = 0.05$.

3. Results

A total of five primer sets were used (Figure 1), corresponding to the five different methods to measure CTG expansion size in our patients. All the primers were located outside the CTG repeat expansion.

The name of each primer and its sequence are shown in Table 1. The length of each PCR product varied from 106 to 324 base pairs, depending on the primer set used (plus the number of CTG repeats for each patient) (Table 1). Thus, the differences in PCR amplification among the techniques were small.

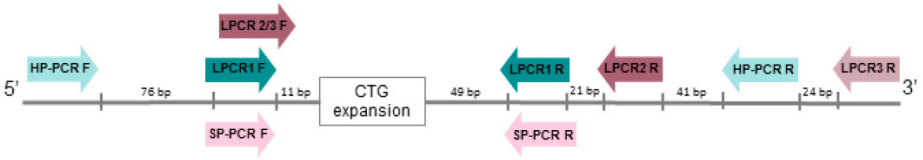

Figure 1. DMPK gene and location of the primer sets. Primers sets are indicated by the name of the technique and identified with a different background color. All of them were located outside the CTG expansion. LPCR2 and LPCR3 share the same forward primer. The distance (bp) between primers is also indicated. Abbreviations: F—forward; HPE-PCR—heat pulse extension-polymerase chain reaction; LPCR—long polymerase chain reaction; R—reverse; SP-PCR—small pool polymerase chain reaction.

Some technical difficulties were found with the HPE-PCR method. Although the technique worked in our hands, the results we obtained were inconsistent (Figure S1A) and thus, not comparable to previously published results [13]. Eight controls amplified smears that were similar to the ones found in patients—the status of control in our analysis is guaranteed, since prior to this analysis, we measured CTG alleles by sequencing. Thus, the cause of these unexpected smears is not apparent because they were unrelated to the CTG expansion in the study controls. As such, these data were excluded from statistical analyses. The results for all the methods, but HPE-PCR, are shown in Figure 2.

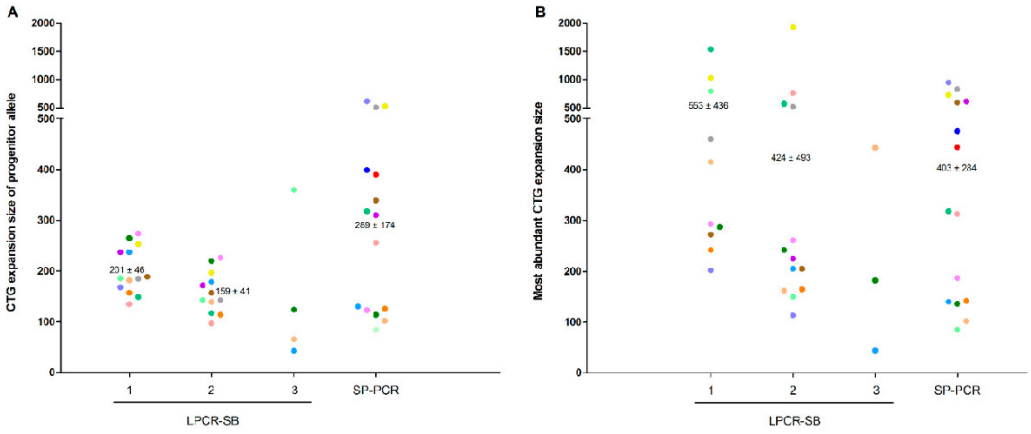

Figure 2. CTG expansion size of the progenitor allele (**A**) and the mode allele (**B**) for each technique. The progenitor allele was estimated by the lowest signal yielded after WT range for LPCR-SB, and by the most frequent lower band present in the samples for SP-PCR. Mode allele was estimated by the more intense signal yielded by LPCR and the most abundant band for SP-PCR. Data for heat pulse extension-polymerase chain reaction are not shown owing to the inconsistency of the results. All valid individual data within the detection limit (2000 CTGs) are shown(different color per patient). Normality was analyzed with the Kolmogorov–Smirnov test and we used a repeated-measures one-way analysis of variance (ANOVA) test for performing within-subject comparisons between methods. No significant group (or 'method') effect was found for the progenitor allele ($p = 0.112$) or the mode allele ($p = 0.653$). Mean and SD values are shown only for those methods included in the within-subject analyses (i.e., LPCR1, LPCR2 and SP-PCR). A significant Pearson correlation was found between the progenitor allele of LPCR1 and LPCR (indicated by an *symbol in the Figure, r = 0.983 [95% confidence interval (CI) 0.940 to 0.996], $p < 0.0001$). Y-axis scale is segmented from 500 CTGs to 2000 CTGs, representing 25% of the total length axis. Abbreviations: LPCR—long polymerase chain reaction; SP-PCR—small pool polymerase chain reaction.

Table 1. Techniques and primer sets used in this study.

Technique	bp of the Amplified Fragment (without CTG Expansion)	Primer Pair	Name	Sequence 5′—3′	Reference
HPE-PCR	324	F	DMKf	GCCAGTTCACAACCGCTCCGAGCGTGGGTC	Orpana et al. [13]
		R	DMKr	ACGCTCCCCAGAGCAGGGCGTCATGC	Orpana et al. [13]
LPCR1-SB	112	F	DM102	GAACGGGGCTCGAAGGGTCCTTGT	Brook et al. [1]
		R	DM101	CTTCCCAGGCCTGCAGTTTGCCCATCCA	Brook et al. [1]
LPCR2-SB	144	F	MDY1D	GCTCGAAGGGTCCTTGTAGCCG	Siciliano et al. [15]
		R	DM1REV	GTGCGTGGAGGATGGAAC	Radvansky et al. [17]
LPCR3-SB	262	F	MDY1D	GCTCGAAGGGTCCTTGTAGCCG	Siciliano et al. [15]
		R	SOMY4R	CGGGTTTGGCAAAAGCAAATTTCCCGA	Musova et al. [19]
SP-PCR	106	F	DM-C	AACGGGGCTCGAAGGGTCCT	Monckton et al. [5]; Gomes-Pereira et al. [18]
		R	DM-DR	CAGGCCTGCAGTTTGCCCATC	Monckton et al. [5]; Gomes-Pereira et al. [18]

Abbreviations: bp—base pairs; F—forward; HPE-PCR—heat pulse extension-polymerase chain reaction; LPCR—long polymerase chain reaction; R—reverse; SP-PCR—small pool polymerase chain reaction.

We also found problems with the LPCR-SB technique, which did not allow amplification of the CTG expansion in some patients (2 for LPCR1-SB and LPCR2-SB, respectively, and 11 for LPCR3-SB). Thus, based on the small amount of individual data points obtained with LPCR3-SB, we also excluded these data from statistical analyses. In LPCR, the amplified product in patients appeared sometimes as a high smear (Figure S1B), probably due to a mobility impairment in long amplifications of highly concentrated DNA (i.e., 100 ng in the final PCR reaction). Attending to the juvenile-classical phenotype of our patients, we established a detection limit of 2000 CTGs. In this context, three CTG sizes of the mode allele in LPCR1-SB were excluded from the study. None of the progenitor expansions surpassed the 2000 CTG-limit. SP-PCR amplified the CTG expansion of all the patients at the first attempt except for two of them—in whom, we had to repeat the amplification in order to correctly detect and quantify CTG expansion. Representative results of SP-PCR can be seen in Figure S1C.

LPCRs yielded shorter progenitor alleles and higher mode alleles compared to SP-PCR (Figure 2). No significant group effect was found with the one-way repeated-measures ANOVA between the three techniques with analyzable data (i.e., LPCR1, LPCR2 and SP-PCR) for the within-subject comparison of CTG expansion size of the progenitor ($p = 0.112$) or mode allele ($p = 0.653$). A significant, strong correlation was found between LPCR1-SB and LPCR2-SB for CTG expansion size of the progenitor allele (r = 0.983 [95% confidence interval (CI) 0.940 to 0.996], $p < 0.0001$). However, no other significant correlation was found for the results obtained with LPCR1-SB, LPCR2 or SP-PCR, respectively (all $p > 0.05$). We further studied possible correlations with age of disease onset, MIRS and mRS scale. We found an inverse, significant correlation between CTG expansion size of the progenitor allele as determined by SP-PCR, and age of disease onset (Figure 3).

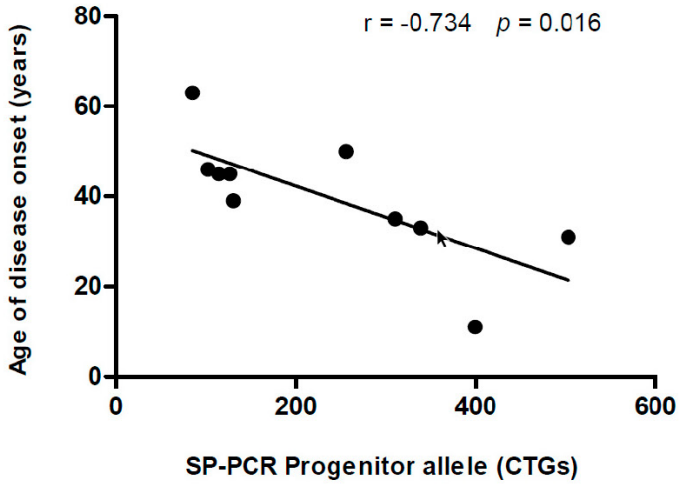

Figure 3. Correlation between the age of disease onset (years) and the CTG expansion size of the progenitor allele obtained through SP-PCR. The 95% confidence interval for the Pearson correlation coefficient was −0.933 to −0.1940 (r = −0.734, $p = 0.016$).

4. Discussion

Although no significant group effect was found with the one-way repeated-measures ANOVA between LPCR1/2 and SP-PCR, our results indicate that there is variability in the number of CTG repeats for a given patient depending on the CTG sizing method. HPE-PCR showed results that were difficult to interpret. Additionally, LPCR3 did not allow amplification of most of the DNAs in the patients. However, the fact that LPCR1 and LPCR-2 did yield some valid results suggests that LPCR-SB might be more sensitive to parameters such as the quality of the input DNA, which is not the case for the SP-PCR technique. SP-PCR was the only technique that enabled amplification of all DNAs

from the patients and in fact, was the only one yielding a result that was correlated with an important phenotype trait of DM1—age of disease onset.

While a strong correlation was found between LPCR1-SB and LPCR2-SB for CTG expansion size of the progenitor allele, no other significant correlation was found. LPCR1/2-PCR yielded lower progenitor sizes and higher mode sizes than SP-PCR. LPCR-SB approaches usually show the expanded alleles as diffuse smears rather than discrete bands, due to the high input of DNA plus the somatic instability of the mutation [20]. This fact hinders differentiation of the progenitor allele size from possible contractions of the repeat. The number of PCR cycles may also affect the results—35 cycles are used in LPCR vs. 28 for SP-PCR—since a high number of cycles facilitates the amplification of shorter products, whereas longer products may be not favored. Moreover, the number of PCR cycles increases PCR slippage, tending to shorten the products. These phenomena could explain that these techniques yielded lower progenitor sizes than SP-PCR. By contrast, SP-PCR—which amplifies only small pools of input DNA—shows discrete bands that allow for a detailed analysis of the mutational spectrum and allele size distribution [18]. As such, this technique enables a better detection and estimation of the progenitor allele from post-contractions of the repeat. In three of the 15 patients, LPCR1-SB yielded some intense signals running high in the gel which, when measured, showed sizes above 2000 CTG repeats. Because the amount of input DNA is high (100 ng) in the different LPCR methods, the DNA mobility in the gel can be impaired, spreading out and yielding a signal that is higher than the actual CTG expansion size. Therefore, when using these LPCR-SB techniques, it would be necessary to set up a threshold for measuring CTG size in the detected smears.

It would be interesting to determine how novel, recently described technologies for CTG sizing [11,12] compare to the methods we assessed here. The sizing kit used by Leferink et al., was based on tripled repeat primed PCR, which is a robust and accurate technique to determine the presence of a CTG expanded allele [11]. However, the sizing of the repeat was limited in their study, set at 180 CTG repeats. In this regard, the most frequent DM1 form, the classical adult form, is usually associated with CTG repeats ranging from fifty to thousands. In fact, more than 70% of the samples in our study had more than 180 CTG repeats. Thus, although the kit reported in the Leferink et al. study would seem very useful for accurate DM1 diagnosis, it would not be suitable to size CTG expansion. In the study by Malbec et al., repeat sizing was performed with a lab-on-chip system that concentrates, separates, and detects DNA fragments in a very short time (actually, less than 5 min) from femtomolar concentrations of PCR-amplified DNAs [12]. Although this system appears as a good alternative to the sizing methods that we assessed, its accuracy would depend on the design of the primers used and the PCR amplification cycle. Furthermore, it would be also necessary to test some PCR designs in order to determine to what extent they are similar. Furthermore, since the chip presented in the Malbec et al. study can detect expansions up to 4Kb, it would have limitations to size samples from patients with congenital DM1. In fact, although their results were promising, only two DM1 blood samples were tested with the new technology, and as such, the interference of somatic mosaicism in CTG sizing remains to be analyzed.

Some studies have described that CTG repeat number can be a good indicator of disease onset [14]. In this regard, we further explored whether the different sizing results were related to the age of disease onset, finding a significant correlation for the SP-PCR method only. These results are overall in accordance with previous studies reporting a correlation between progenitor allele length measured by SP-PCR and both age of disease onset and clinical severity [10,21], although we found no correlation with MIRS or mRS. Progenitor allele length is the major modifier of age of disease onset, and as such, it is very important to use an accurate method for its determination. Concerning the lack of correlation with MIRS and mRS scales, CTG sizes in blood may be poor representatives of muscle status. Thus, future research in this field might study CTG in muscle cells.

5. Conclusions

Our study suggests that, besides the somatic mosaicism caused by CTG repeat instability and the inherent technical difficulties in assessing CTG expansion, there is overall heterogeneity among the different methods that are currently available, which makes it difficult to rely on them as valid predictors of disease phenotype. International agreement is needed to determine which is the most suitable methodology to characterize CTG expansion size in patients with DM1.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4425/11/7/757/s1. Figure S1: Representative results of the three different methods: HP-PCR, LPCR-SB and SP-PCR. (A) HP-PCR gel. Three patients and three controls are shown as representative results of HP-PCR. Controls showed similar signals to the ones found in patients. As such, these data were excluded from statistical analysis. (B) LPCR-SB gel showing the amplification results of the three primer sets: (1) DM102 and DM101 (LPCR1); (2) MDY1D and SOMY4R (LPCR2); and (3) MDY1D and DM1rev (LPCR3). Three patients and one control are shown. With this technique, we could not amplify the CTG expansion in some patients (two for LPCR1-SB and LPCR2-SB, respectively, and 11 for LPCR3-SB). The amplified product in patients appears as high smears, probably due to highly concentrated DNA (100 ng). (C). SP-PCR gel. Two patients and one control are shown, with four replicates per sample. Using small amounts of input DNA (300 pg), the technique allows us to study single genomic equivalents, which are amplified individually and further separated in the gel. Therefore, the gel shows discrete bands corresponding to individually CTG tracts of different lengths.

Author Contributions: A.B.-L., I.L.-P., E.K. and S.A.C. carried out the experiments. J.N.-M., M.A., G.L. and A.A. contributed to sample preparation. A.R.-F., A.M.-P., G.P.-M. and J.C.-C. performed sample collection. G.N.-G. and A.M.-P. conceived the experiments and supervised the project. A.B.-L. performed the interpretation of the results and wrote the manuscript. G.N.-G. and Alejandro Lucia supported and helped in writing the manuscript. D.G.M., S.A.C., and A.L. provided critical feedback and helped shape the research. J.J.M. and N.M.M.-M. contributed to the experimental procedures. All authors have read and agreed to the published version of the manuscript.

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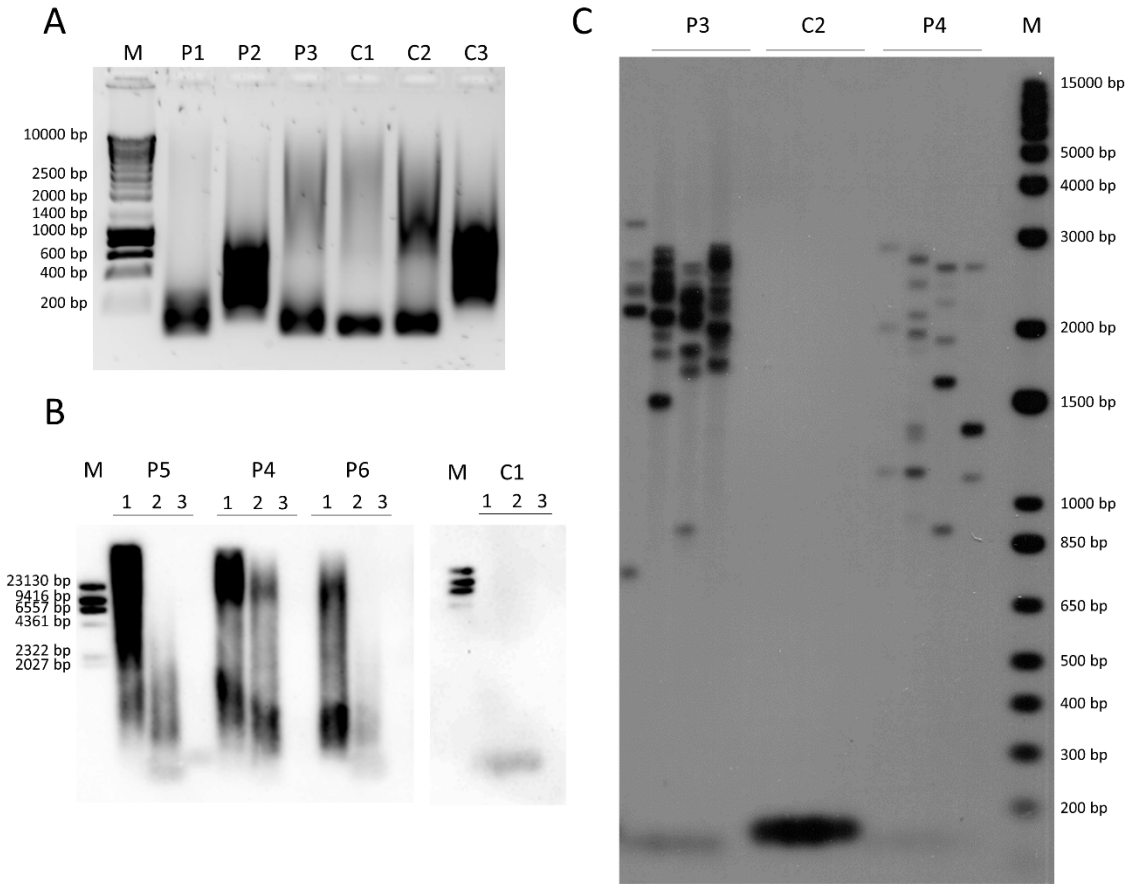
Supplementary Materials



Chapter II

Preliminary findings on CTG expansion determination in different tissues from patients with myotonic dystrophy type 1

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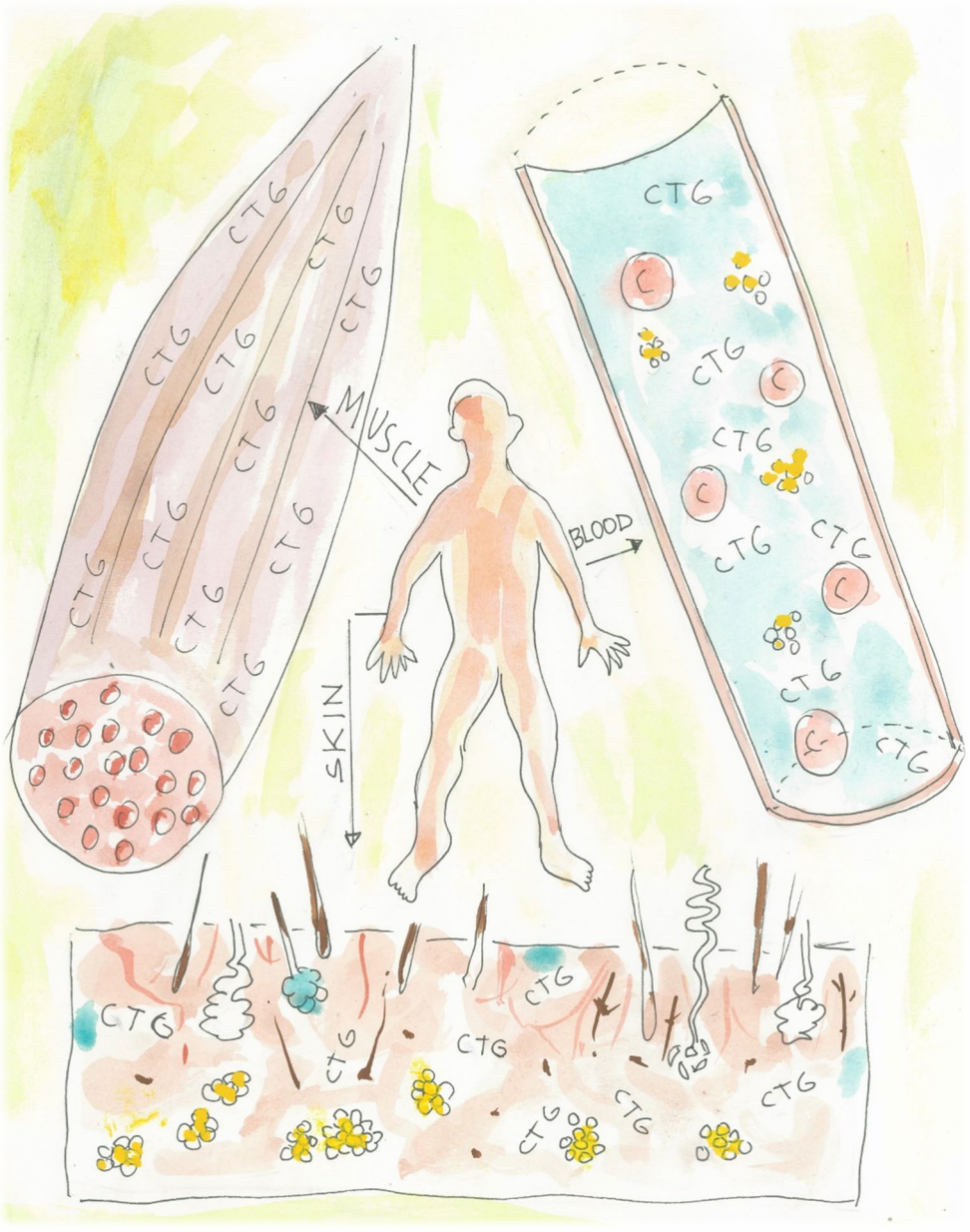
This paper is under review



Brief Report

Preliminary findings on CTG expansion determination in different tissues from patients with myotonic dystrophy type 1

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ABSTRACT: Myotonic Dystrophy type 1 (DM1) is characterized by a high genetic and clinical variability. Determination of the genetic variability in DM1 might help to determine whether there is an association between CTG expansion and the clinical manifestations of this condition. We studied the variability of the CTG expansion (progenitor, mode, and longest allele, respectively, and genetic instability) in three tissues (blood, muscle and tissue) from eight patients with DM1. We also studied the association of genetic data with the patients' clinical characteristics. Although genetic instability was confirmed in all the tissues that we studied, our results suggest that CTG expansion is larger in muscle and skin cells compared with peripheral blood leukocytes. While keeping in mind that more research is needed in larger cohorts, we have provided preliminary evidence suggesting that the estimated progenitor CTG size in muscle could be potentially used as an indicator of age of disease onset and muscle function impairment.

Keywords: Myotonic Dystrophy Type 1; somatic instability; CTG expansion; blood; muscle; skin

INTRODUCTION

Myotonic dystrophy type 1 (DM1) is caused by a CTG expansion in the 3' untranslated region of the *dystrophia myotonica*-protein kinase (*DMPK*) gene [1]. The CTG expansion is highly unstable, showing size variation both within [2] and between tissues [3–6]. Genetic instability hinders the establishment of genotype/phenotype correlations in patients with DM1, and most studies assessing CTG expansion have focused solely on blood samples [7–10]. Here we used small pool polymerase chain reaction (SP-PCR) to study CTG expansion in three different tissues from affected patients. We estimated the progenitor allele, the mode of CTG expansion size and

the highest CTG repeat number, as well as the genetic instability of the CTG repeat (*i.e.*, the difference between the progenitor and the mode CTG size) in the different tissues. We also analyzed the potential association between the different CTG measures, on one hand, and patients' clinical phenotype, on the other.

RESULTS

We studied eight patients (six women). The patients' cohort included five unrelated individuals and three sisters from the same family (P3, P4 and P8). Symptom onset occurred during adulthood in seven patients (with symptoms starting around their fifties in two of them (P2, aged 48 years; P7, 50 years)) whereas in one patient the symptoms started earlier in life (P1, 15 years) (**Table 1**). One patient (P7) carried previously reported CCG interruptions [11]. All the patients had clinical myotonia, but only two showed a mild impairment in biceps muscle strength (as reflected by a score of 4 in the 0 to 5 Medical Research Council (MRC) scale). Performance in the 6-minute walking distance test averaged 377 meters (range 251–519). When using the muscular impairment rating scale (MIRS), 25% of patients showed minimal signs of muscular impairment, 50% had distal weakness and 25% had mild-moderate proximal weakness. Most patients (87.5%) were independent in daily life activities (score of 0-2 on the modified Rankin (0 to 6) scale), and only one (P5) had a moderately severe disability (4).

1 Table 1. Clinical characteristics of the patients.

Patient	Sex	Age of symptom onset (years)	Biceps muscle (MRC scale)	Myotonia (seconds)	6-minute walking distance (meters)	MIRS	mRS
P1	**F**	**15***	**4**	**0.52**	**348**	**4**	2
P2	M	48	5	0.67	251	3	2
P3	F	36	5	0.73	368	2	1
P4	F	42	5	0.98	338	3	1
P5	F	27	4	NP	NP	4	4
P6	M	36	5	0.96	519	3	2
P7	F	50	5	NP	436	2	1
P8	F	35	5	NP	NP	3	2

1. Abbreviations: F, female; M, male; MRC, Medical Research Council NP, not performed. Symbol: * although it was not possible to determine the actual age of disease onset of this patient, since at the first visit (age 36) she had obvious signs that commonly appear early in patient's life (including oval pallor and temporal atrophy) we considered that the disease onset occurred during adolescence.

The estimated progenitor, most abundant and longest CTG expansion size were measured in blood (n=8), muscle (n=7) and skin samples (n=5) (**Fig. 1**). An example of CTG expansion determination is shown in **Suppl. Fig. 1**. DNA from one muscle biopsy and from three skin biopsies yielded no amplification and therefore precluded CTG sizing. We found no differences across tissues for progenitor CTG (p=0.449 for tissue effect with the repeated-measures one-factor ANOVA), mode size (p=0.247), and genetic instability (p=0.691). By contrast, a significant

(p=0.041) tissue effect was found for the longest CTG with significant differences (p<0.05) found for all post hoc pairwise comparisons (thus, mean of the longest CTG size in blood (665 CTGs) < muscle (1110 CTGs) < skin (1408 CTGs). In blood samples, we found the following significant correlations (all p<0.05): blood progenitor CTG size *vs* mode allele, r=0.900 (95% confidence interval 0.536–0.982); mode allele *vs* longest allele, r=0.805 (0.231–0.963); and longest allele *vs* progenitor allele, r=0.861 (0.398–0.975). These results suggested that the progenitor, the mode and the longest expansion size were uniformly distributed, with the CTG tract evenly expanded in blood. For muscle and skin samples, a significant (p<0.05) correlation was only found between the progenitor and mode size (muscle: r=0.769 (0.037–0.964); skin: r=0.895 (0.061–0.993)). No correlation was found between tissues. Finally, as P7 carried variant repeats, we compared the results of this patient with those of patients carrying pure repeats to determine whether P7 showed a more stable CTG repeat behavior in any of the tissues, which was not the case (**Suppl. Fig. 2**).

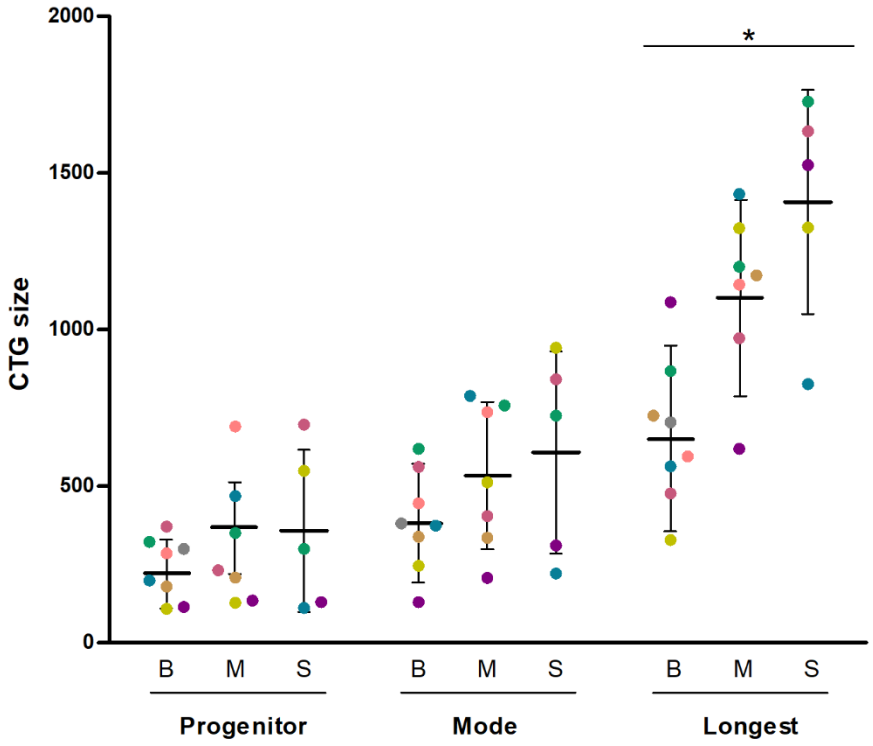

Figure 1. CTG repeat number estimates of the progenitor, mode, and longest allele length in the study patients. All the individual CTG data are shown, with each circle representing one single CTG size (using a different color per patient). The mean and SD values of the different CTG measures are also shown. Symbol: * p=0.041 for tissue effect with repeated-measures one-factor ('tissue') ANOVA.

We further studied the relationship between genetic and clinical data and found a significant (p<0.05) correlation between the progenitor allele found in muscle and both age of disease onset (r=-0.850 (-0.977–0.268)) and the MRC corresponding to the studied muscles (r=-0.932 (-0.992–0.496)) (**Fig. 2**). The CTG mode length in muscle was also correlated with the MRC score for the muscle in question (r=-0.898 (-0.989 to -0.319), p<0.05). By contrast, no significant correlation was found between CTG expansion in blood or skin and the clinical manifestation of the disease.

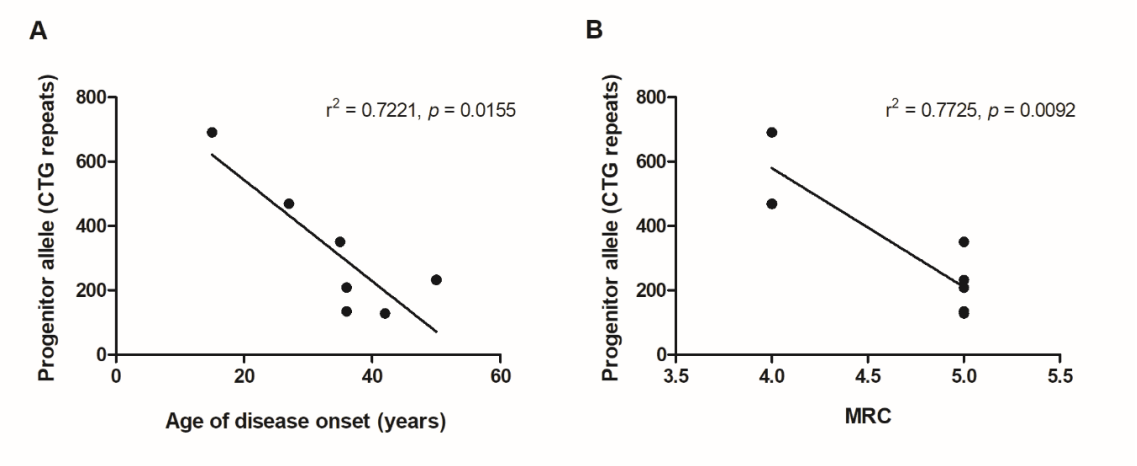

Figure 2. Correlations between the progenitor CTG size present in muscle and the age of disease onset and the MRC of the studied muscles. (A) Correlation between the progenitor CTG size present in muscle and the age of disease onset (r=-0.850 [-0.977–-0.268], p<0.05). (B) Correlation between the progenitor CTG size present in muscle and the MRC of the studied muscles (r=-0.932 (-0.992–-0.496), $p < 0.05$).

DISCUSSION

Our preliminary results suggest that CTG expansions might be in general larger in muscle and skin than in blood. Previous studies have reported that *i)* patients' muscle fibers carry larger expansions than peripheral blood leukocytes [3–5], and *ii)* patients' fibroblasts carry larger expansions than peripheral blood lymphocytes[6]. However, no previous study has assessed CTG repeats in blood, muscle and skin cells from the same patients. Furthermore, we studied in depth the CTG expansion size by applying SP-PCR methodology and took into account the genetic instability of CTG expansion (instead of focusing on one single CTG size per tissue using a less accurate southern blot-based analysis). This strategy allowed us to determine that the progenitor and the mode size did not differ significantly across tissues, as opposed to the highest expansion. This finding suggests that the CTG tract is expanding in different manners in each tissue.

The three tissues presented genetic instability. The complex phenomenon of genetic instability can be produced by numerous mechanisms, including not only DNA repair mechanisms but also DNA replications, transcriptions, and epigenetic changes. In muscle and skin, a non-dividing cell status coupled with DNA repair mechanisms might play an important role in producing genetic instability. In the case of blood cells, the CTG expansion instability could also be affected by the division status of these cells.

No differences in the CTG stability were found between P7 (who carried variant repeats) and the rest of the patients (who had pure expansion repeats in blood, skin and muscle). Some authors have shown a stabilizing effect of the variant repeats [12,13], which was not confirmed in P7. Although the case of P7 might be an exceptional one, no conclusions can be really drawn as our study is the first to analyze genetic instability in different tissues of a patient with variant repeats.

When analyzing each tissue independently, we found that all measures (progenitor, mode and highest CTG) were correlated to each other in blood samples, suggesting that the progenitor CTG size leads the genetic instability of CTG expansion in blood. Conversely, in muscle and skin

the progenitor was correlated with the mode but not with the highest CTG, suggesting that the genetic instability of the CTG tract is more random in these tissues, probably due to the longer length of CTG repeats.

We found that the progenitor allele in muscle tissue was the only CTG variable associated with age of disease onset. This finding is not surprising when considering that the muscle is one of the most affected tissues in patients with DM1. In this regard, we studied samples mostly from biceps muscle, whereas disease manifestation might start earlier in the *tibialis* anterior muscle [14,15].However, some authors have hypothesized that CTG in muscle tissue could show a stronger correlation with disease severity than CTG determined in blood cells [4,5], for which we were actually able to provide preliminary ('proof-of-concept') evidence. Furthermore, our data also suggest that the CTG size in muscle is associated with patients' muscle impairment (as determined with the MRC scale). These results might reflect a close relationship between CTG expansion in muscle and the degree of functional affectation in this tissue. Although it has been shown that the progenitor size determined in peripheral blood leukocytes of patients with DM1 is also a good indicator of age of disease onset [16], we failed to find a correlation between blood measures and age of symptom onset, maybe owing to the small sample size of our study. Previous studies in larger cohorts of patients with DM1 have in fact reported a close relationship between CTG size in blood and cardiac complications [17] or survival [18].

In conclusion, we found preliminary evidence for the presence of genetic instability in all the patients' tissues that we studied, yet with muscle and skin cells carrying larger expansions than peripheral blood leukocytes. Although more research is needed in larger cohorts, our preliminary data suggest the estimated progenitor CTG size as determined in muscle tissue is associated with age of disease onset and muscle functional impairment.

MATERIALS AND METHODS

This study was approved by the local ethics committee (University Hospital Germans Trias i Pujol, # PI15-009) and was performed in agreement with the Declaration of Helsinki for Human Research. All participants signed an informed consent. The study included eight patients with DM1 and eight controls with no previous family history of neuromuscular disorders (recruited from the traumatology department in whom surgery was needed. DM1 diagnosis was confirmed or discarded with triplet primed-PCR[11] in all the study participants. Clinical information of DM1 patients was obtained from the medical records and updated in the last visit.

We obtained three different samples from patients and controls: blood, muscle biopsy and skin biopsy. All samples were obtained at the same time. Blood was collected in EDTA tubes and frozen at -20ºC before DNA extraction. The muscle biopsy was obtained from the left biceps muscle in all individuals except for one patient (P8, *vastus lateralis* muscle). Skin biopsy was obtained with a 0.5cm skin punch. Muscle biopsies were frozen immediately and stored at -80ºC before DNA extraction. Skin biopsies were first seeded in plates with human serum and gelatin 1.5% (1:2) and cultured with DMEM supplemented with 5% of Fetal Bovine Serum and PSF 1x, at 37ºC for 6 days, and frozen afterwards at -80ºC before DNA extraction.

Genomic DNA was isolated from peripheral blood [19]. Genomic DNA was extracted from muscle and skin tissue by homogenization in 100mM Tris-HCl, pH 7.8, and 5mM EDTA until these tissues were disaggregated. Thereafter tissues were digested in 20mg/mL proteinase K and 10% SDS for 16h at 37ºC, and treated with 5.5 M NaCl, phenol and chloroform isoamyl (1:24) before DNA precipitation with isopropanol. DNA quality and quantity were measured by Qubit Fluorometric (Themo Fisher Scientific; Waltham, MA) and Agilent 4200 TapeStation analysis (Agilent Technologies; Santa Clara, CA).

To measure CTG expansion size, SP-PCR was performed [20]. Briefly, small amounts of input DNA (300 pg) were used with the flanking primers DM-C and DM-DR as previously described [20]. We used custom PCR Master Mix (Thermo Fisher Scientific; Waltham, MA) supplemented with 69 mM 2-mercaptoethanol, and Taq polymerase Thermus aquaticus (Sigma-

Aldrich; Gillingham, UK) at 1 unit per 10 μL, supplemented with 5% DMSO and the annealing temperature was 63.5°C. DNA fragments were resolved by electrophoresis on a 1% agarose gel, followed by Southern blot [21]. The estimated CTG sizes (the progenitor, the mode and the longest CTG size) in each tissue were determined by comparison against the molecular weight ladder, using GelAnalyzer 19.1 software. We studied four replicates of each sample, allowing the analysis of the allele distribution CTG sizes, since the most representative allele sizes that are present in a given sample are shown in the gel (see **Supplementary Fig. 1** for more information on the different measurements). Genetic instability was calculated by subtracting the progenitor CTG size from the mode CTG size amplified for each sample. This method has been optimized by Prof. Monckton's group [20].

We used repeated-measures, one-factor (*i.e.*, 'tissue') analysis of variance (ANOVA) to compare CTG variables (progenitor, mode, longest repeat length, and somatic instability) across the different tissue samples within each subject. When a main tissue effect was found, post hoc pairwise comparisons (skin *vs* blood, skin *vs* muscle, and blood *vs* muscle) were done with the Bonferroni test. We also determined the relationship between the aforementioned CTG variables and patients' clinical characteristics with Pearson's correlations (or Spearman correlations for those data that were not normally distributed, as determined with the Shapiro-Wilk test). The level of significance was set at 0.05 (two-tailed).

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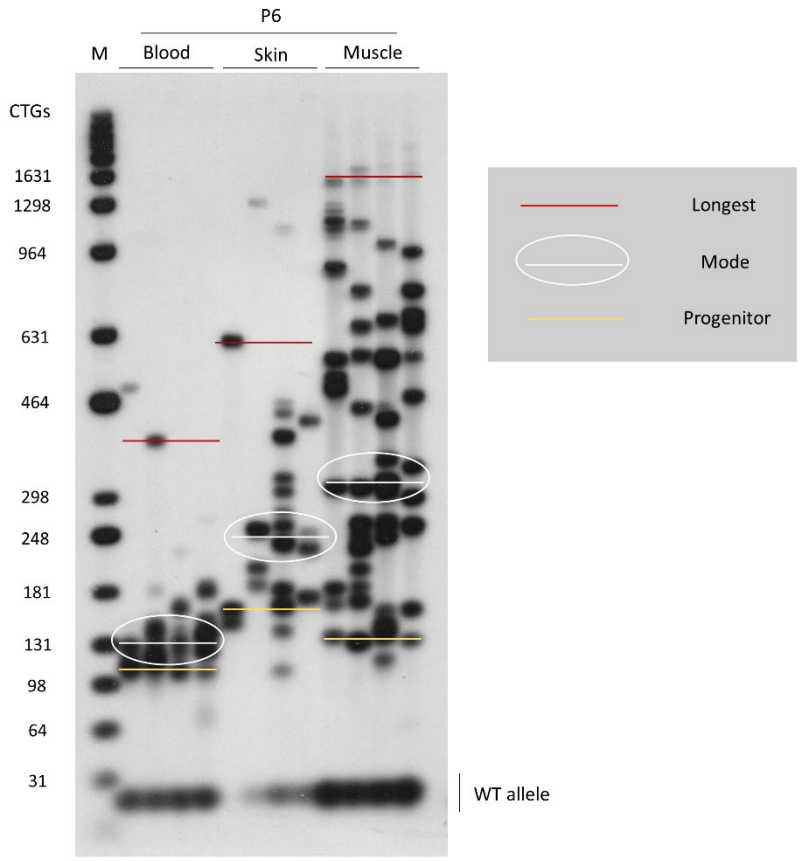
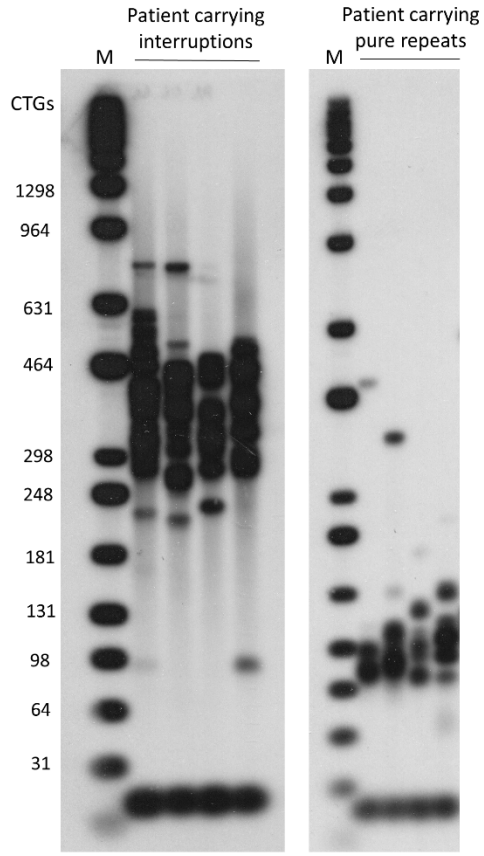
Supplementary Materials



Supplementary Figure 1. Representative SP-PCR gel with the different CTG size measurements. Analysis of the CTG repeat number estimates of patient P6 in blood, muscle and skin. Progenitor allele size (yellow), presumable inherited from the previous generation, is measured considering the shortest CTG size that is more represented in the four replicates. The estimated mode allele size (white) is measured based on the most abundant bands present in the four replicates. The longest CTG size (red) is calculated considering the highest intense signal present in the sample. M = molecular weight ladder; WT= wild type.



Supplementary Figure 2. CTG size allele distribution in blood of the patient carrying interruptions vs one patient carrying pure repeats. M = molecular weight ladder.

Chapter III

A DM1 Family with Interruptions Associated with Atypical Symptoms and Late Onset but not with a Milder Phenotype

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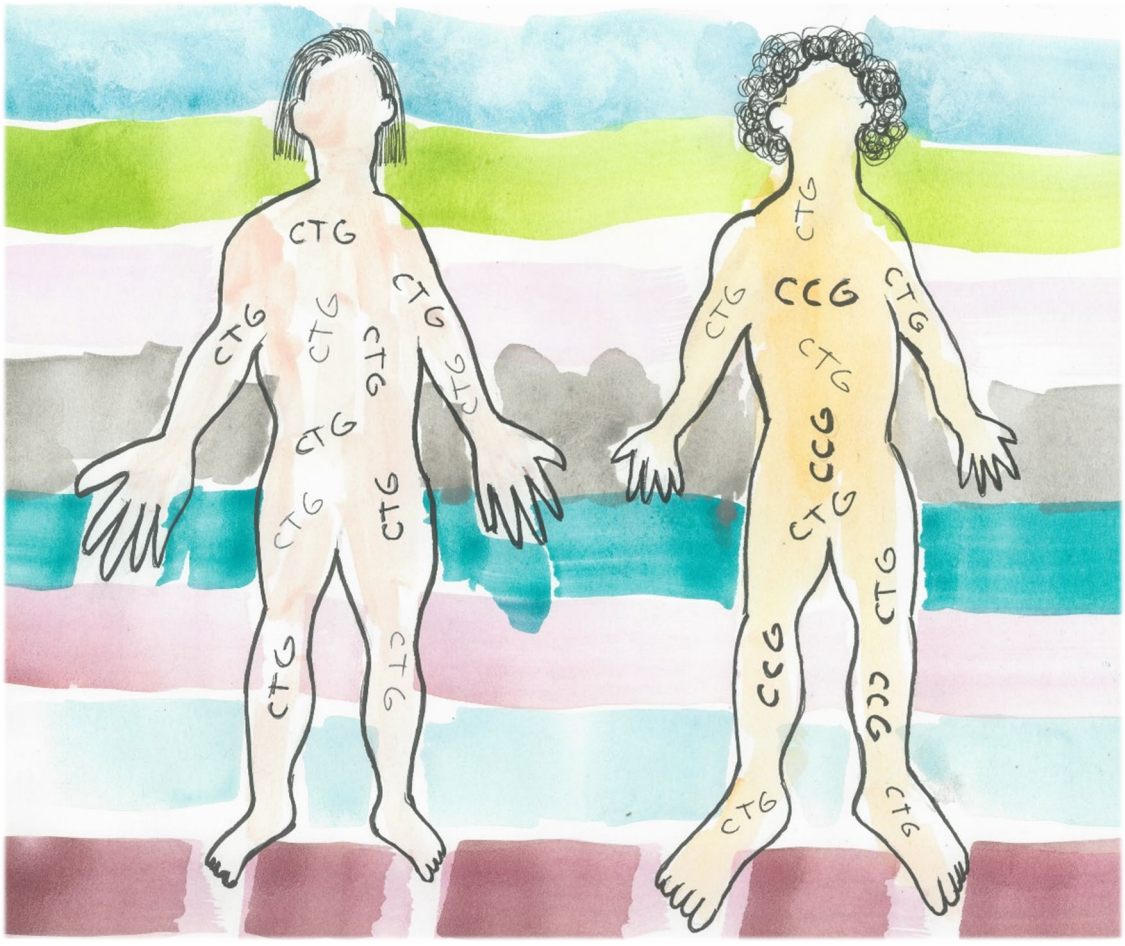

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RESEARCH ARTICLE

Human Mutation HGVS HUMAN GENOME VARIATION SOCIETY WILEY

A DM1 family with interruptions associated with atypical symptoms and late onset but not with a milder phenotype

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Abstract

Carriage of interruptions in CTG repeats of the myotonic dystrophy protein kinase gene has been associated with a broad spectrum of myotonic dystrophy type 1 (DM1) phenotypes, mostly mild. However, the data available on interrupted DM1 patients and their phenotype are scarce. We studied 49 Spanish DM1 patients, whose clinical phenotype was evaluated in depth. Blood DNA was obtained and analyzed through triplet-primed polymerase chain reaction (PCR), long PCR-Southern blot, small pool PCR, *Aci*I digestion, and sequencing. Five patients of our registry (10%), belonging to the same family, carried CCG interruptions at the 3′-end of the CTG expansion. Some of them presented atypical traits such as very late onset of symptoms (> 50 years) and a severe axial and proximal weakness requiring walking assistance. They also showed classic DM1 symptoms including cardiac and respiratory dysfunction, which were severe in some of them. Sizes and interrupted allele patterns were determined,

*Alfonsina Ballester-Lopez and Emma Koehorst contributed equally to this work.

and we found a contraction and an expansion in two intergenerational transmissions. Our study contributes to the observation that DM1 patients carrying interruptions present with atypical clinical features that can make DM1 diagnosis difficult, with a later than expected age of onset and a previously unreported aging-related severe disease manifestation.

KEYWORDS
atypical symptoms, interruptions, late onset, myotonic dystrophy type 1, severe phenotype, Steinert disease, variant repeats

1 | INTRODUCTION

Myotonic dystrophy type 1 (DM1, Steinert disease; MIM# 160900) is a multisystemic disorder with an overall estimated prevalence of 1:8000 (Harper PS, 2001), being the most common form of inherited muscular dystrophy in adults. DM1 patients show wide phenotypic heterogeneity, not only in the age of onset but also in severity and type of clinical manifestation. DM1 patients can be broadly divided into five subtypes based mainly on their age of onset: congenital (<1 month), childhood (1 month–10 years), juvenile (10–20 years), adulthood/classic (20–40 years), or late onset (>40 years; De Antonio et al., 2016). Classic DM1 symptoms include muscle weakness, myotonia, respiratory failure, cardiac conduction defects, cataracts, and endocrine disturbances. The younger subtypes, congenital and childhood onset, are characterized primarily by cognitive and learning abnormalities (Douniol et al., 2012; Meola & Cardani, 2015).

DM1 is an autosomal dominant disorder caused by a CTG expansion in the 3′ untranslated region of the myotonic dystrophy protein kinase (*DMPK*) gene. Unaffected individuals carry 5–35 CTG repeats whereas individuals carrying between 35 and 50 repeats are usually asymptomatic. Yet in the latter, *DMPK* alleles have a higher mutation rate and are labeled as "pre-mutational alleles" (Imbert, Kretz, Johnson, & Mandel, 1993). The length of the CTG expansion varies widely between patients, ranging from 50 to thousands of CTGs and has been associated with age of symptom onset and severity (Groh et al., 2011; Logigian et al., 2004). A CTG repeat size ≤150 CTGs, ≤1,000 CTGs and >1000 CTGs is common to late onset, adulthood/classic and congenital DM1, respectively (Meola & Cardani, 2015). However, a high individual variability exists among DM1 patients of the same subtype and thus caution is needed when using CTG expansion length to predict disease progression. For instance, congenital cases have been found with CTG repeat lengths clearly below 1,000 CTG repeats (Tsilfidis, MacKenzie, Mettler, Barceló, & Korneluk, 1992) and late onset DM1 cases have been reported with over 1,000 CTG repeats (Clark, Petty, & Strong, 1998). Another feature of the disease that makes it difficult to infer potential genotype/phenotype correlations is the presence of "somatic mosaicism." Indeed, the CTG expansion is highly unstable in both germline and somatic cells, and this instability persists through the lifetime of the patient. Thus, the CTG repeat size of a given patient represents the mean value for different CTG repeat sizes, which in turn can vary depending on the age at which the patient is studied. These potential confounders for sizing CTG repeat makes it difficult to find genotype-phenotype correlations for DM1. In this respect, estimating the inherited allele length has proven to be a more accurate predictor of potential genotype-phenotype correlations in this disease. (Higham, Morales, Cobbold, Haydon, & Monckton, 2012; Morales et al., 2012).

Because the aforementioned fact that CTG expansion instability is also present in germline cells, new alleles with different CTG repeat sizes are constantly generated and children may inherit CTG repeat sizes considerably longer than those found in the transmitting parent. This leads to the so-called "anticipation" phenomenon, which occurs in DM1 and in other triplet disorders, and is characterized by the fact that the disease may develop earlier in life in each successive generation (Harper, Harley, Reardon, & Shaw, 1992). In DM1, the sex of the transmitting parent plays an important role in anticipation, although both paternal and maternal transmission have been described. The paternal allele seems more unstable and leads more frequently to higher expansions in offspring, especially with CTG expansions below 100 repeats (Ashizawa et al., 1994). However, very large expansions causing congenital DM1 are transmitted almost exclusively by affected mothers (Harley et al., 1993), with few exceptions reported (Di Costanzo et al., 2009; Zeesman, Carson, & Whelan, 2002). In contrast, the congenital form is frequently observed after transmission from mothers who are carriers of more than 500 CTG repeats. Contractions of the CTG expansion upon transmission have also been reported, with a higher estimated prevalence in paternal transmission compared with maternal transmissions (6.7 vs. 19.5%; López de Munain et al., 1996).

In most cases, the CTG expansion in expanded *DMPK* alleles is an uninterrupted sequence. However, in the last decade, pathological variant expansions containing unstable CCG, CTC, GGC, and CAG sequence interruptions at the 3′ and 5′ ends of the *DMPK* allele have been reported, with a prevalence of 3–5% among DM1 (Botta et al., 2017; Braida et al., 2010; Cumming et al., 2018; Musova et al., 2009; Pešović et al., 2017; Santoro et al., 2013; Tomé et al., 2018). In addition, intergenerational transmissions typically lead to smaller CTG expansions when compared with noninterrupted DM1 families, suggesting a stabilizing effect of the expansion on germline transmission (Botta et al., 2017; Pešović et al., 2017; Tomé et al., 2018). These findings might also explain why no congenital cases have been described in maternal transmission of interrupted alleles.

Most of the phenotype consequences of interruptions remain poorly understood and vary considerably between studies, ranging from a complex neurological phenotype to a later age of onset (Botta et al., 2017; Braida et al., 2010; Cumming et al., 2018; Santoro et al., 2015; Musova et al., 2009; Pešović et al., 2017). There is an urgent need to determine the phenotypes that associate with the subset of DM1 patients presenting with interruptions. This information is required for patient management, genetic counseling and future clinical trials. In the literature, only a few families and some isolated cases have been described, and their reported clinical data are scarce. In the present study, we have analyzed a large cohort of Spanish DM1 patients belonging to several families. Our aim was to identify DM1 patients carrying variant repeats and to perform an in-depth analysis of their clinical phenotypes. This might help to gain insight into the modifying effect that these repeat interruptions could have in DM1 diagnosis, clinical manifestation, and patient follow-up.

2 | MATERIALS AND METHODS

2.1 | Editorial policies and ethical considerations

This study was approved by the ethics committee of the University Hospital *Germans Trias i Pujol* (ref. PI-15–129) and was performed in accordance with the Declaration of Helsinki for Human Research. Written informed consent was obtained from all the participants.

2.2 | Participants

Forty-nine DM1 patients belonging to 36 different families who were evaluated in our center during the 2015–2018 period participated in this study. Clinical and genetic information was collected and stored in a secure registry. Their clinical phenotype was evaluated by the neurologists of our team. Muscle strength was assessed using the manual Medical Research Council (MRC) scale. The most recent ophthalmological, cardiological and respiratory examinations carried out by the corresponding specialists were reviewed, as well as blood analyses, electrocardiograms, echocardiograms, and functional respiratory and swallowing tests. Functional status and disability were assessed using the Muscular Impairment Rating Scale (MIRS), the modified Rankin Scale (mRS), and the Rasch-Built Myotonic Dystrophy type 1 activity and participation scale (DM1-Activ).

2.3 | DNA extraction and bidirectional triplet-primed polymerase chain reaction (TP-PCR)

Total genomic DNA was extracted from peripheral blood samples, as previously described (Miller, Dykes, & Polesky, 1988). To assess the size and the presence of interruptions in the expanded allele, all DM1 blood DNA samples were analyzed by bidirectional TP-PCR. TP-PCR was performed with primers DM1for-FAM, DM1-CAG-rev, and P3 at the 5′-end of the CTG expansion, or DM1rev-FAM, DM1-CTG-for, and P3 at the 3′-end of the CTG expansion, as previously described by Radvansky, Ficek, Minarik, Palffy, and Kadasi (2011). Both TP-PCRs (5′ and 3′) were performed with 100 ng of genomic DNA, 10× PCR Buffer containing 15 mM of $MgCl_2$, 10 nM of dNTP mixture, 0.5 U of TaKaRa DNA polymerase (TaKaRa, Kusatsu, Japan), 3% dimethyl sulfoxide (DMSO), and 0.2 μM of each primer. PCR amplification conditions were the same for both TP-PCRs: initial denaturation at 94°C for 5 min, followed by 34 cycles at 94°C for 1 min, 65°C for 1 min, and 72°C for 2 min and a final extension step at 72°C for 7 min. Correct amplification was assessed on a 2% agarose gel. PCR products were separated on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, EEUU) and data were analyzed with PeakScanner Software v1.0 (Applied Biosystems).

2.4 | *AciI* digestion and Southern blot

We used a digestion with *AciI* and Southern blot-long PCR strategy to determine the presence of interruptions of the CCG/CGG type. DNA (100 ng) was amplified using the primers MDY1D-F GCTCGAA GGGTCCTTGTAGCCG and DM1-rev GTGCGTGGAGGATGGAAC. The conditions of the long PCR were as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s and annealing-extension at 65°C for 7 min. The final extension was performed at 65°C for 10 min. Fifty microliters of long PCR products were divided into two parts, one digested with *AciI* and the other not digested. An aliquot (10 μl) of each sample was resolved in an agarose gel and the products were detected by Southern blot hybridization. A DIG-labeled LNA probe (5′-gcAgCagcAgCagCagcAgca-3′, with lower and upper-case letters representing an unmodified and an LNA nucleotide, respectively) was used to detect the expansions through chemiluminescence.

2.5 | Sequencing

To determine the pattern of the interruptions we first amplified the DNA using primers GC1_CC, GC1_CCG, P2-rev, and P3, as described elsewhere (Pešović et al., 2017). Products were resolved in a 3% agarose gel and purified using QIAquick gel extraction kit (Werfen, Barcelona, Spain). Purified products were sequenced with BigDye™ Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequences were analyzed with Chromas version 2.6.2. The *DMPK* gene reference sequence used was NG_009784.1.

2.6 | Small pool PCR and *AciI* digestion

To estimate the length of the expanded progenitor allele (ePAL), small-pool PCR (SP-PCR) was carried out using flanking primers DM-C and DM-DR as previously described (Gomes-Pereira, Bidichandani, & Monckton, 2004; Monckton, Wong, Ashizawa, & Caskey, 1995). PCR was performed using Custom PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, EEUU) supplemented with 69 mM 2-mercaptoethanol, and Taq polymerase (Sigma-Aldrich, Gillingham, UK) at 1 unit per 10 μl. All reactions were supplemented with 5% DMSO and the annealing temperature was 63.5°C. DNA fragments were resolved by

electrophoresis on a 1% agarose gel, and Southern blot hybridized as described (Gomes-Pereira et al., 2004; Monckton et al., 1995). Autoradiographic images were scanned and ePAL estimated from the lower boundary by comparison against the molecular weight ladder, using CLIQS 1D gel analysis software (TotalLab, Newcastle upon Tyne, UK). To analyze again the presence of CCG or CGG variant repeats, an additional step was added to the SP-PCR protocol. PCR products were purified using the QIAquick (Qiagen, Venlo, the Netherlands) PCR purification kit and split into two aliquots, one of which was digested with *Aci*I. They were then resolved and blotted as before.

3 | RESULTS

3.1 | Clinical phenotypes

Five of 49 DM1 patients (~10%) were found to have interruptions in the 3′-end of the CTG expansion. They belonged to the same family (Figure 1). Patient P1, P2, and P3 are sisters who paternally inherited the disease, and patient P4 is the son of P2 (Figure 1) whereas patient P5 is the daughter of P3. A summary of their clinical characteristics is shown in Table 1.

Patient P1 is the oldest of the siblings, and currently the most severely affected of all five patients. The first symptom she reported was a weakness at the age of 52. Subsequently, she developed a generalized weakness, which interfered with her ability to cope with daily life activities. We studied her when she was 72-year-old and the most striking feature upon clinical examination was a severe axial weakness with dropped-head. The patient also presented with mild weakness in the upper and lower limbs, with only little myotonia. Another remarkable fact was that she had moderate facial weakness, but almost no ptosis and no temporal atrophy. She also presented with bilateral cataracts, dysphagia for liquids and frontal baldness. She had a heart pacemaker implanted since the age of 71 and used nocturnal noninvasive mechanical ventilation, and had no cognitive impairment.

Patient P2: Symptoms started at the age of 50 with mild fatigue and myotonia. At the moment of inclusion in the study (aged 62), the clinical examination revealed the only mild weakness of the neck flexor muscles, with mild handgrip myotonia and minimum ptosis. Complementary explorations showed a first-degree atrioventricular block and low values of maximum inspiratory and expiratory pressure (38% and 22% of normal, respectively), and of both forced vital capacity (81%) and expiratory volume in 1 s (96%). The patient presented with bilateral cataracts and severe baldness. No limb weakness, dysphagia or cognitive impairment was found.

Patient P3: The first sign reported was handgrip myotonia in her fifties. At the moment of examination (age 60) she also had a severe axial weakness with mild proximal limb weakness and moderate distal weakness. Like her oldest sister (Patient P1), she had a moderate facial weakness with no ptosis or temporal atrophy. She also presented with bilateral cataracts, frontal baldness, and dysphagia for liquids. Cardiological studies revealed a first-degree atrioventricular block. No respiratory or cognitive involvement was found.

Patient P4: This male patient (aged 35 years) carrying an interrupted allele was asymptomatic upon clinical examination and had no detectable myotonia or cardiac alterations.

Patient P5: She was diagnosed at the age of 25 based on the family history, although clinical manifestation did not start until 2 years later, starting with handgrip myotonia. At the moment of assessment (age 32) she presented with mild neck flexor and facial weakness and handgrip and percussion myotonia, without limb weakness. She has a first-degree atrioventricular block and cataracts, but no respiratory impairment.

3.2 | Molecular analysis of interruptions

Interrupted alleles were first detected as gaps in the pattern of contiguous peaks detectable by capillary electrophoresis by 3′ TP-PCR (Figure 2). Patients P2 and P4 showed a similar interruption pattern, whereas P1, P3, and P5 showed different interruption patterns (Figure 2). No alterations were found with 5′ TP-PCR (Figure S1).

We performed an *Aci*I digestion of PCR products to test for the presence of CCG or GGC variant repeats in patients P1–P5. In all these patients, the results showed a downward shift of the smear in the gel of the digested product compared with the nondigested product (Figure 3). This indicated that *Aci*I had cleaved the PCR product and the interruption was likely either a CCG or a GGC triplet. In addition, since the bidirectional TP-PCR performed in the entire cohort was limited to the outer regions of the CTG expansion, we performed an *Aci*I digestion in the entire DM1 cohort to search for possible undetected CCG or GGC interruptions in the middle

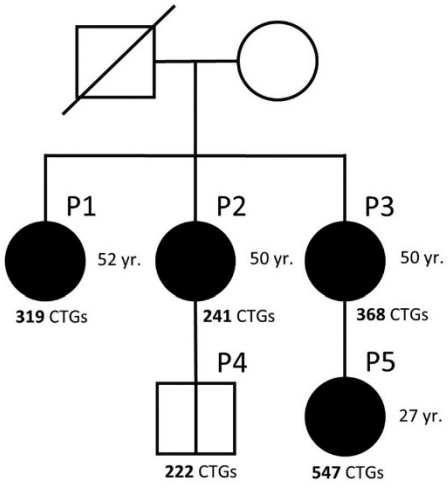

FIGURE 1 Pedigree of the interrupted patients in our cohort. CTG, number of repeats in CTG; P1, patient P1; P2, patient P2; P3, patient P3; P4, patient P4; P5, patient P5. The father of P1, P2, and P3 died (sudden cardiac death)

TABLE 1 Clinical characteristics of the interrupted cases

	P1	P2	P3	P4	P5
Sex	**Female**	**Female**	**Female**	**Male**	**Female**
Age of onset (years)	52	50	50	Asymptomatic	27
Age of assessment (years)	72	62	60	35	32
Cardiopathy	Pacemaker	1st degree AV-block	1st degree AV-block	None	1st degree AV-block
Respiratory disturbance	Yes, nocturnal NMV	Alteration MIP and MEP	None	None	None
Dysphagia	Liquids	No	Liquids	None	None
Cognitive impairment	None	None	None	None	None
Cataracts	Yes	Yes	Yes	None	Yes
Metabolic disturbance	None	None	Hypothyroidism	None	None
Myotonia	Yes	Yes	Yes	None	Yes
Polyneuropathy	None	None	None	None	None
CK level	Normal	Normal	213 U/L	Normal	ND
Limb weakness					
Facial ptosis	Yes	Mild	Yes	None	Mild
Flexor/extensor neck	1 (Dropped head)	4	2	5	5
Axial weakness	Severe	None	Severe	None	None
Upper limb proximal (MRC)	4	5	4	5	5
Upper limb distal (MRC)	4	5	3	5	5
Lower limb proximal (MRC)	4	5	4	5	5
Lower limb distal (MRC)	4	5	3	5	5
6 MWT (m)	250	436	240	658	800
MIRS	4	2	4	1	2
mRS	3	1	3	0	2
DM1-Activ	23	37	23	40	39

Abbreviations: 6 MWT, six-minute walking test; AV, atrioventricular; CK, creatine kinase; DM1-Activ, Rasch-built myotonic dystrophy type 1 activity and participation scale; MEP, maximum expiratory pressure; MIP, maximum inspiratory pressure; MIRS, Muscular Impairment Rating Scale; mRS, modified Rankin Scale; MRC, Medical Research Council; ND, not determined; NMV, noninvasive mechanical ventilation.

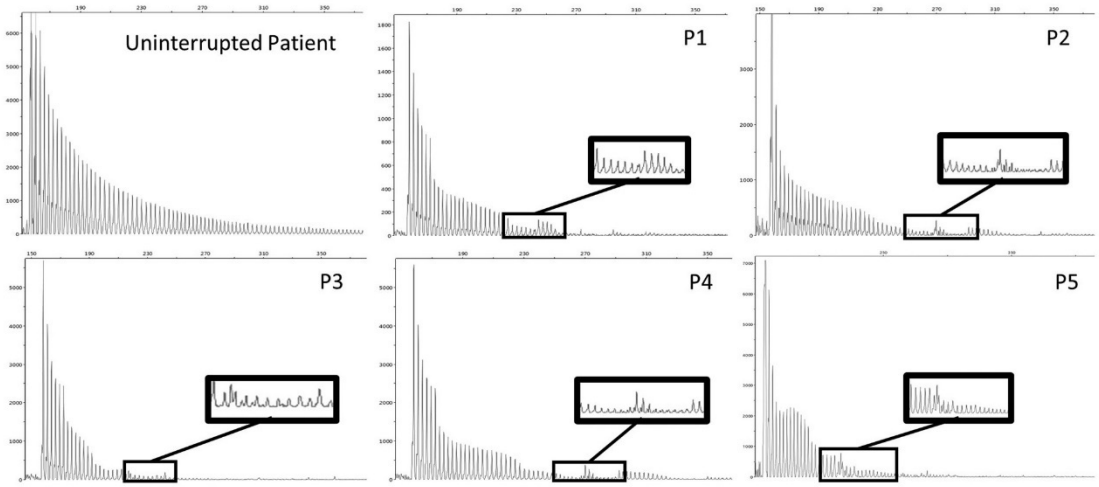

FIGURE 2 Peak scan results of triplet-primed polymerase chain reaction (TP-PCR) of the 3′-end, obtained with DNA extracted from blood. Interruptions indicated by black box. P1, patient P1; P2, patient P2; P3, patient P3; P4, patient P4; P5, patient P5

region of the CTG expansion. No additional CCG or GGC interruptions were found in our 44 remaining DM1 patients.

Sequencing revealed the presence of several CCG interruptions in the CTG expansion of our five patients carrying interrupted alleles (Figure 4). The pattern of CCG interruptions was identical in the mother (patient P2) and son (P4), but different between all the other family members (patients P1, P3, and P5). In P1, we found some isolated CCG repeats scattered across the expansion. Patients P2 and P4 showed a complex CCG pattern, with one pair of CCGs together with other isolated CCG repeats. Patient P3 had a few CCGCTG hexamers, but inside of a more complex pattern including CCG interruptions in other positions. Patient P5 (the daughter of P3) showed a pattern similar to that of her mother with respect to the hexamers, but with some extra CCGs located in different positions, generating three consecutive CCG repeats. During the sequencing process, we purified different bands from the same patient to assess the influence of somatic instability in the interruption pattern (Figure 4b). In the different bands analyzed, the same pattern was observed in each patient.

SP-PCR (Figure 5) provided information on the repeat size of the ePAL for some of the patients: P1, 319 CTGs; P2, 241 CTGs; P4, 222 CTGs; P5, 547 CTGs. The expansion range due to the instability of the repeat was also determined: P1, 319–900 CTGs; P2, 241–651 CTGs; P4, 222–332 CTGs; and P5 = 547 to 897 CTGs. For patient P3, the expanded allele did not amplify well under these conditions, so it was not possible to determine the ePAL or expansion range. This may be due to the specific pattern of variant repeats present. However, in the SP-PCR we could amplify 368 CTGs, which was the only sizing of the expanded allele that we could make. Additionally, the type of interruptions was analyzed through *Aci*I digestion in SP-PCR experiments, which again showed that the interruptions were of the CCG type (data not shown). By comparing the range of these bands, we could determine a contraction in the repeat size from patient P2 to P4 (i.e., from mother to son) but expansion from patient P3 to P5 (i.e., from mother to daughter). This expansion was also linked to anticipation, with an early age of onset for P5 when compared with her mother (P3).

4 | DISCUSSION

The effect of variant repeat patterns on the DM1 clinical phenotype is still unclear. On one hand, this genetic alteration has been shown to be associated (albeit in one family only) with a complex co-segregated neurological phenotype, including an intermediate Charcot–Marie–Tooth neuropathy, early hearing loss and encephalopathic attacks (Braida et al., 2010). In contrast, variant repeats have been associated with a milder or atypical phenotype, including a later age of onset (Cumming et al., 2018; Musova et al., 2009; Pešović et al., 2017), a DM2-like muscle phenotype (Pešović et al., 2017), as well as with an absence of muscular dystrophy (Musova et al., 2009) or central nervous system symptoms (Santoro, Masciullo, Silvestri, Novelli, & Botta, 2017). These reports have led to a tendency to believe that patients with interrupted alleles have some atypical symptoms, but overall a milder phenotype than their age-matched DM1 noninterrupted peers with a similar repeat length. In this respect, we had the unique possibility to study a family containing interrupted cases of whom three were aged above 60 years. In this regard, although our data were obtained in a small number of patients within the same family, our results support the occurrence of atypical DM1 features and late age of onset, but not of a milder phenotype in patients carrying interruptions.

Despite the fact that several of the classical symptoms of DM1 could be found in the three sisters—such as myotonia, cataracts, and cardiopathy—some peculiarities need to be highlighted. An atypical trait was the distribution pattern of muscle weakness in two of the sisters. Indeed, besides the distal limb weakness commonly found in DM1 patients, these two sisters presented with proximal limb weakness and severe axial involvement. One of them also had a dropped-head, which resembled limb-girdle muscle dystrophy and severely affected her ability to perform activities of daily living. Another atypical trait of these patients is that they did not have the typical myopathic face expected in DM1 patients, despite the presence of moderate facial weakness. Although the interrupted cases showed several classic DM1 symptoms, the presentation of atypical symptoms could interfere with (and thus delay) the diagnosis.

Based on the algorithm published by Morales et al (Morales et al., 2012), the ePAL of patients P1, P2 and the expanded allele size of P3 should be theoretically associated with an age of onset around 30 s whereas in our patients symptoms did not actually start until they were in their 50 s. In this regard, it should be first noted that it is very difficult to assess the age of onset in DM1 patients. The definition of age of onset refers to the age at which an individual starts to develop one or more clinical features or symptoms of a disease. In actual clinical practice, this depends on the capacity of the patient to report such symptoms or to remember the time when they started, and also

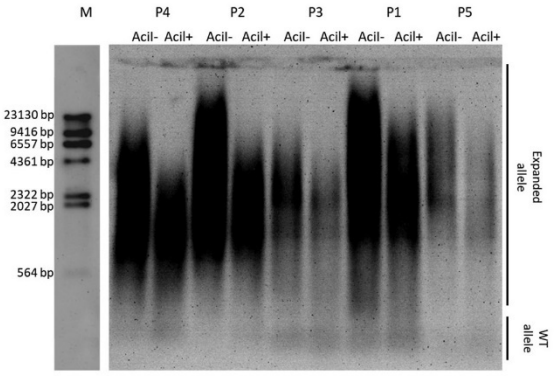

FIGURE 3 Southern blot of long polymerase chain reaction (PCR) products from patients carrying variant repeats. For each patient, we show two conditions: digestion with (+) and without (−) the enzyme *Aci*I (recognizing the pattern CCGC). bp, base pairs; M, molecular weight marker; P1, patient P1; P2, patient P2; P3, patient P3; P4, patient P4; P5, patient P5; WT, wild type

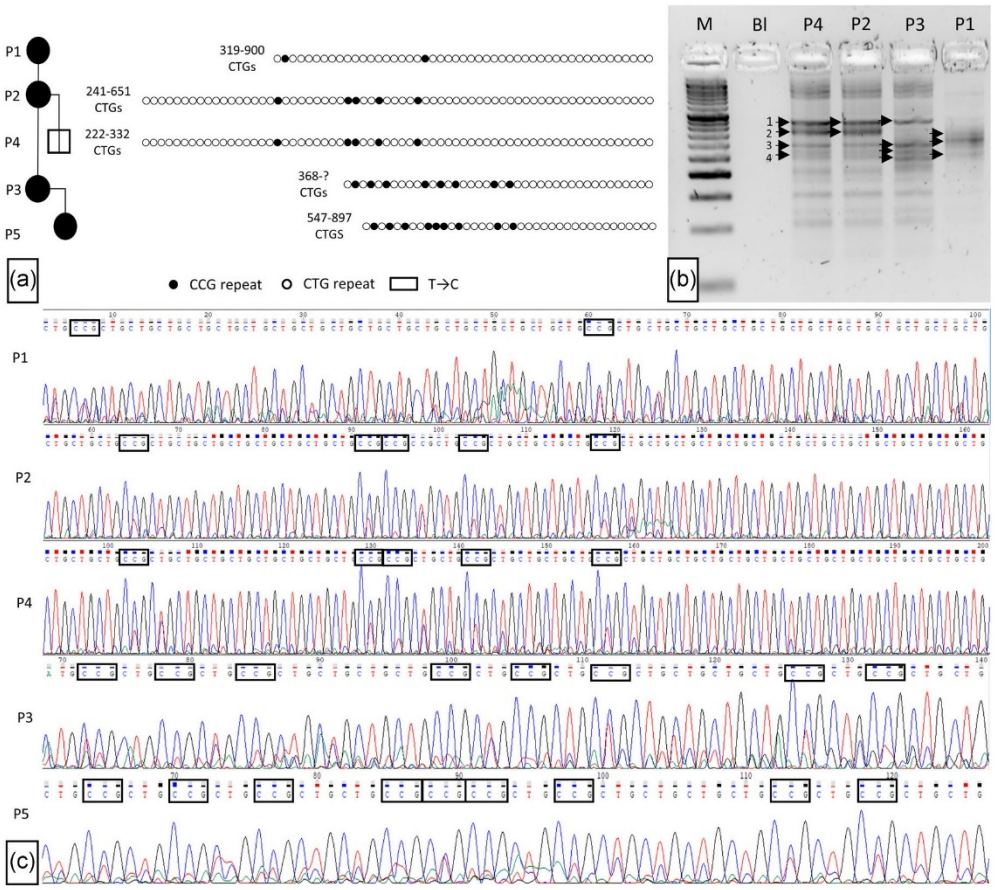

FIGURE 4 Sequencing the interrupted alleles. (a) Schematic structure of *DMPK* expanded alleles of the interrupted DM1 family in our study. CTG repeats are shown in white, CCG repeats in black. Indicated size ranges were estimated by Southern blot analysis. (b) Cutting and purifying strategy for several bands of polymerase chain reaction product (indicated by arrows), from each interrupted patient, which are affected by somatic instability. (c) Sequences showing the CCG interruptions are marked by black rectangles. Bl, PCR reaction with no DNA; M , molecular weight marker; P1, patient P1; P2, patient P2; P3, patient P3; P4, patient P4; P5, patient P5. The *DMPK* gene reference sequence used was NG_009784.1

on the ability of the physician to recognize them. Thus, the reported age of onset can be quite variable, depending on which symptoms are searched for by the physician and on the patient's own reports. Our patients P1–P3 (the three sisters) reported their first symptoms in their 50s (being myotonia, and difficulty to walk, the first abnormalities that made them suspect they had a major medical condition). Patient P4 was a 35-year-old and was still asymptomatic. Based on his ePAL length (222 CTGs), he should show a classic DM1 phenotype (Morales et al., 2012), but no signs could be detected upon neurological examination. This late onset of symptoms has been previously reported in interrupted DM1 families and seems to be a fingerprint for most of the cases (Botta et al., 2017; Cumming et al., 2018; Musova et al., 2009; Pešović et al., 2017).

In the family we studied, anticipation was observed in one of the two intergenerational transmissions that we assessed, since in the other intergenerational transmission one of the patients (P4) was still asymptomatic. In patient P5 (whose first symptom was myotonia, at the age of 27), we found a bigger size of the expansion and an earlier age of onset than her progenitor. Although this anticipation in interrupted families has been previously reported (Pešović et al., 2017), after reviewing all the published families (Table 2), we assessed anticipation in every single reported family. In the rest of intergenerational transmissions reported, and in the case of our patients P2 and P4, anticipation could not be assessed since patients in the next generation are still asymptomatic. The explanation for these findings is not apparent, since anticipation is not expected in these families; indeed, interruptions are thought to be related to a stabilization or even contraction of the pathological expansion (Braida et al., 2010; Cumming et al., 2018; Musova et al., 2009; Pešović et al., 2017; Tomé et al., 2018). However, anticipation was found in our studied intergenerational transmission, with this finding being also reported in other interrupted DM1 patients based on reported age of onset (Table 2). In our family, no

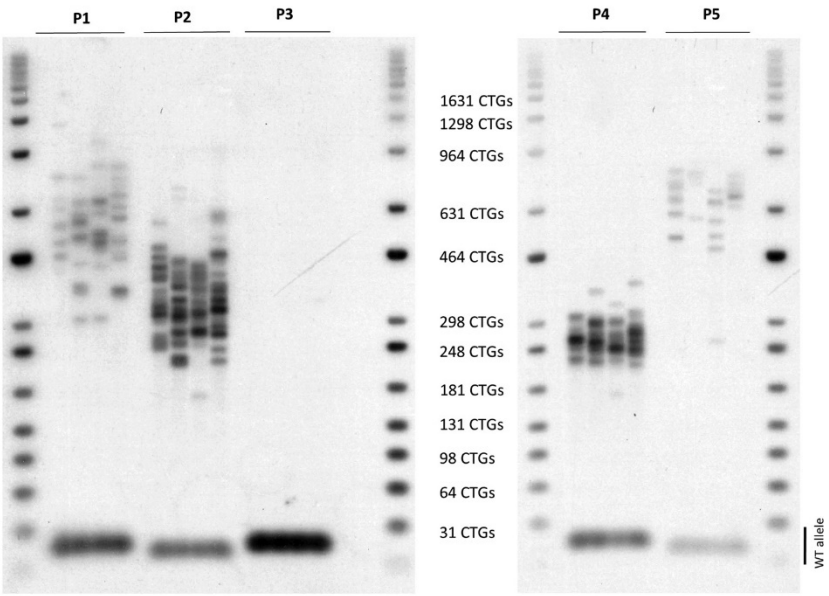

FIGURE 5 Small pool polymerase chain reaction from patients carrying variant repeats. For each patient, several lines show the normal and the expanded alleles. CTGs, number of repeats; P1, patient P1; P2, patient P2; P3, patient P3; P4, patient P4; P5, patient P5; WT allele, wild type allele

congenital, childhood or juvenile cases of DM1 were observed. Among the interrupted families reported in the literature (Table 2), at least three juvenile DM1 cases (age < 18 years) have been described (Braida et al., 2010; Pešović et al., 2017), but no congenital or childhood case. Thus, the absence of infantile DM1 seems also to be a distinctive trait for interrupted expansions.

The prevalence of interrupted alleles among our patients was ~10%, and 3% among the studied DM1 families. This is in overall agreement with previous studies in which the prevalence in families ranged from 3% to 5% (Botta et al., 2017; Braida et al., 2010; Musova et al., 2009; Pešović et al., 2017). The type of interruption present in our cohort was CCG, which is currently the most frequently reported variant repeat. However, the difficulties we experienced in characterizing the pattern of interruptions in our family members must be emphasized, with such difficulties mainly due to a technical limitation of TP-PCR and sequencing, which have a limited ability to detect interruptions deeper inside the expansion. In addition, characterization is affected by the PCR slippage and by somatic mosaicism (implying more noise in readouts and thus a higher difficulty to identify the interrupted pattern). Our sequences showed in some cases double peaks of C and T at the same position, and we decided to consider only those interruptions where the C peaks were above T in the electropherograms, which might have resulted in the loss of CCG interruptions in our patients' sequences. We sequenced several amplified bands coming from the same TP-PCR to determine whether somatic mosaicism was also affecting the pattern of CCG interruptions, but the same patterns were found in all the sequences. Despite the aforementioned limitations, we determined the interrupted pattern in all the studied family members. We observed a substantial change in the interruption pattern in every transmission, and the number and position of the CCGs changed in every generation, except for one intergenerational transmission. Thus, interruption patterns can be conserved or vary upon transmission. Both situations have been previously described in interrupted families (Musova et al., 2009; Pešović et al., 2017; Tomé et al., 2018). Our CCG interruptions were found in blocks of two or three (in hexamers of CCGCTG that were repeated two or three times), and also as isolated cases. Due to technical limitations, we cannot be certain that other interruptions are not present deeper in the CTG expansion. TP-PCR and sequencing allowed us to study the flanking regions of the CTG expansion, but the middle part remained undetected. We detected the contraction of the expansion between patients P2 and P4, but expansion between patients P3 and P5. Previous studies suggest that CTG expansion containing variant repeat patterns display more frequently stable, or even contracted, *DMPK* alleles instead of further expanded *DMPK* alleles (Cumming et al., 2018; Musova et al., 2009; Pešović et al., 2017; Tomé et al., 2018). However, some studies have also found the expansion of the interrupted alleles from one generation to the other (Braida et al., 2010; Cumming et al., 2018; Pešović et al., 2017). Perhaps these expansions are less frequent than in pure CTG expansions transmission, but they do occur. Therefore, caution is needed with genetic counseling with regard to prospective parents with DM1.

Our study contributes to the observation that DM1 patients carrying interruptions may have atypical symptoms that can make the diagnosis of DM1 difficult, with a later age of onset and a previously

TABLE 2 Analysis of the literature reported interrupted families

Article	Cases	Patient code	Age S	Age O	Relationship	Anticipation	Atypical findings in the examination	CTG repeats number	Type of interruption
Musova et al. (2009)	3 families								
	Family A	A-1 (Fetus)	0		Fetus of A2			230	CTC and CCG
		A-2	31	–	Daughter of A4	NPS		300	CTC and CCG
		A-3	23	–	Daughter of A4	NPS		400–500	CTC and CCG
		A-4	54	40 s	Brother of A5			600–800	CTC and CCG
		A-5	53	40 s	Sister of A4		Electromyography confirmed myotonia but no dystrophy	450–650	CTC and CCG
		A-6	29	–	Daughter of A5	NPS		600–750	CTC and CCG
		A-7	31	–	Son of A5	NPS		270	CTC and CCG
	Family B	B-1	50	40 s	Father of B2		No muscle weakness or muscle atrophy	450	CCG
		B-2	25	–	Son of B2	NPS		400	CCG
	Family E	E-1	20	1 year[a]	Son of E2		Upper limbs showed no muscle atrophy, weakness or myotonic reaction	43	CCG
		E-2	56	–	Father of E1	A		43	CCG
Braida et al. (2010)	1 family								
	Family 1	III-9	55	25	Cousin of III-17 and III-16		Charcot–Marie–Tooth disease, acute encephalopathy, and early hearing loss	229^{P}	CCG and GGC
		III-16	57	44	Sister of III-17		Charcot–Marie–Tooth disease, acute encephalopathy, and early hearing loss	170^{P}	CCG and GGC
		IV-19	37	20	Son of III-16	A	Charcot–Marie–Tooth disease, acute encephalopathy, and early hearing loss	213^{P}	CCG and GGC
		IV-20	34	24	Son of III-16	A	Charcot–Marie–Tooth disease	213^{P}	CCG and GGC
		III-17	61	35	Sister of III-16		Charcot–Marie–Tooth disease, acute encephalopathy, and early hearing loss	179^{P}	CCG and GGC
		IV-21	30	17#	Son of III-17	A	Charcot–Marie–Tooth disease	220^{P}	CCG and GGC
		IV-22	28	25	Daughter of III-17	A	Charcot–Marie–Tooth disease	225^{P}	CCG and GGC
Botta et al., 2017	3 families								
	Family A	A1	66	58	Father of A2		Absence of myotonia and cataracts	1,000–1,400	CCG
		A2	39	31	Daughter of A1	A	Absence of muscle weakness	475–640	CCG
		A3	0	N/A	Fetus of A2			500	CCG
	Family B	B1	55	51	Mother of B2			740–930	CCG
		B2	28	–	Dauther of B1	NPS		450–550	CCG
	Family C	C1	58	58	Mother of C2		Absence of muscle weakness and cataracts	140	CCG
		C2	40	37	Daughter of C1	A	Absence of muscle weakness and cataracts	121	CCG
		C3	0	N/A	Fetus of C2			113	CCG
Pešović et al. (2017)	3 families								
	Family DF1	DF1-1	57	39	Mother of DF1-2 and DF1-3			520–1,250	CCG
		DF1-2	37	30	Son of DF1-1	A	Absence of: percussion myotonia, ptosis, cataracts, and muscle wasting. Presence of calf hypertrophy, suggesting DM2	370–730	CCG

(Continues)

TABLE 2 (Continued)

Article	Cases	Patient code	Age S	Age O	Relationship	Anticipation	Atypical findings in the examination	CTG repeats number	Type of interruption
		DF1-3	30	15#	Son of DF1-1	A	Calf hypertrophy	450–970	CCG
	Family DF2	DF2-1	45	40	Father of DF2-2		Similar involvement of both proximal and distal muscles and winging scapulae in the right side.	320–600	CCG
		DF2-2	14	12#	Daughter of DF-1	A	Mild ptosis and mild percussion myotonia	200–240	CCG
	Family DF5	DF5-2	27	22	Sister of DF5-3		Normal strength of the sternocleidomastoid muscle and very mild myotonia	250–350	CTC
		DF5-3	22	21	Sister of DF5-2			300–620	Noninterrupted
Cumming et al. (2018)	3 families								
	Family 1	14	25.5	–	Daughter of 165	NPS	Absence of muscle weakness, myotonia, and cataracts	381^{ePAL}	CCG
		57	20.5	5	Son of 165	A		597^{ePAL}	Noninterrupted
		165	59	28	Brother of 83			383^{ePAL}	Noninterrupted
		83	46	38	Brother of 165			105^{ePAL}	Noninterrupted
	Family 2	182	35.5	–	Brother of 184	NPS	Absence of muscle weakness, mild masseter myotonia and peripheral membrane irritability on EMG.	293^{ePAL}	CCG
		184	28	20	Brother of 182			288^{ePAL}	Noninterrupted
		206	70	60	Father of 182 and 184			90^{ePAL}	Noninterrupted
		242	65	ND	Sister of 206			80^{ePAL}	Noninterrupted
	Family 3	15	39	–	Daughter of 234	NPS	No clinical apparent weakness or myotonia and no cataracts	303^{ePAL}	CCG
		54	40	35	Brother of 15			146^{ePAL}	Noninterrupted
		234	ND	ND	Father of 15 and 54			496^{ePAL}	Noninterrupted
Our study	1 family								
	Family 1	Patient 1	72	52	Sister of Patient 2 and 3		Severe axial weakness with dropped-head. Mild weakness in upper and lower limb muscles, with only little myotonia. Moderate facial weakness, almost no ptosis, and no temporal atrophy	319^{ePAL}	CCG
		Patient 2	62	50	Sister of Patient 1 and 3		Mild weakness of the neck flexor muscles, no limb weakness, and minimum ptosis	241^{ePAL}	CCG
		Patient 3	60	50	Sister of Patient 1 and 2		Severe axial weakness, mild proximal limb weakness, and moderate distal weakness. Moderate facial weakness with no ptosis or temporary atrophy	368	CCG
		Patient 4	35	–	Son of Patient 3	NPS		222^{ePAL}	CCG
		Patient 5	32	27	Daughter of Patient 2	A	Mild neck flexor and facial weakness, but no limb weakness.	547^{ePAL}	CCG

Abbreviations: –, asymptomatic; A, anticipation; Age S, age of Sampling; Age O, age at Onset; ePAL, estimated progenitor allele; N/A, not applicable; ND, no data; NPS, not possible to establish yet.
[a]Authors clarify in the paper that he had isolated symptoms, cannot be considered childhood DM1.

unreported aging-related severe disease manifestation. Indeed, some of our older patients needed mechanical ventilation and a pacemaker, and besides their cardiorespiratory problems, they had muscle weakness with subsequent impairment in daily life activities and walking ability. Despite the small sample size of our study sample, our results challenge the notion that interrupted patients who remain asymptomatic until their late 30s or 40s are not at risk for having a severe phenotype later in life. Indeed, our patients developed a classical DM1 phenotype after their 50s. These patients require clinical follow-up and genetic counseling similar to noninterrupted DM1 patients. In the family we studied, we found some characteristics that add to the current body of knowledge regarding interrupted families: a later age of onset, variation of CCG repeat pattern between intergenerational transmission, anticipation due to the earlier age of onset of symptoms in next generation and no cases of congenital or childhood onset of DM1. In addition, we have found other previously undescribed characteristics, such as a predominant axial weakness. However, the small number of interrupted patients present in the DM1 population makes it hard to perform genotype-phenotype correlations and there is still much uncertainty. Studies with larger DM1 cohorts, preferably with DM1 families, are needed to unravel the phenotypic consequences of variant repeat patterns and to study their effect on intergenerational transmissions of the *DMPK* expanded allele.

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CONFLICTS OF INTEREST

G. P.-M reports personal honoraria from Shire, and Sanofi-Genzyme, outside the submitted work. D. G. M. has been a scientific consultant and/or received honoraria or stock options from Biogen Idec, AMO Pharma, Charles River, Vertex Pharmaceuticals, Triplet Therapeutics, LoQus23, BridgeBio, Small Molecule RNA and Lion Therapeutics and he also had a research contract with AMO Pharma. The remaining co-authors declare no conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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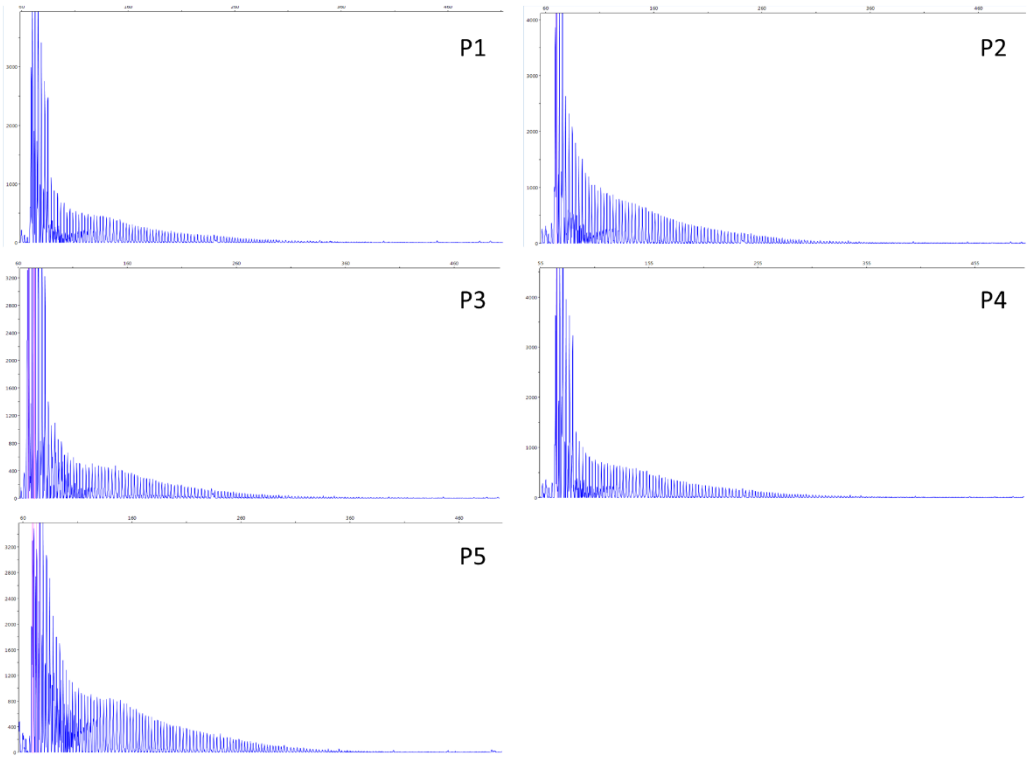
SUPPORTING INFORMATION



Supp. Figure S1: 5’ TP-PCR analysis for the patients of this study.

Chapter IV

Three-dimensional Imaging in Myotonic Dystrophy Type 1

Linking Molecular Alterations with Disease Phenotype

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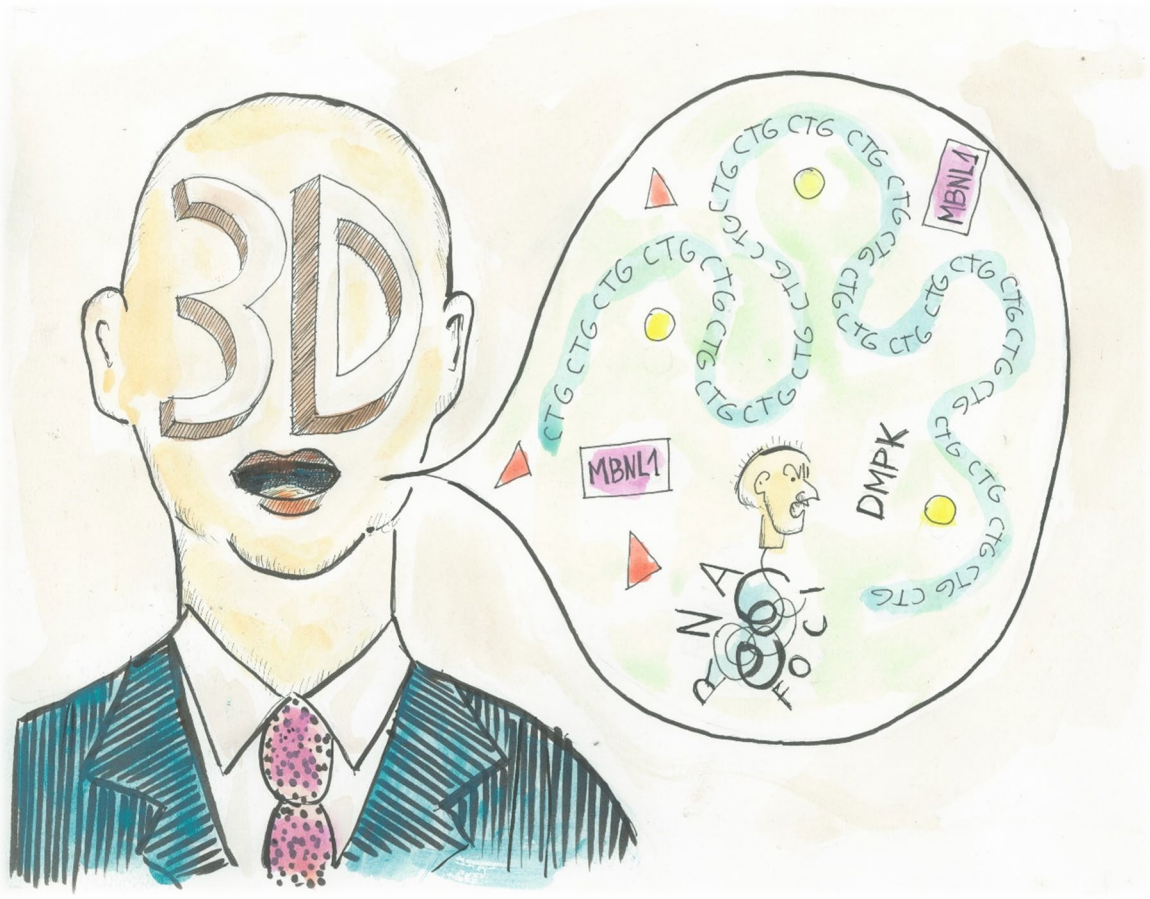

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Three-dimensional imaging in myotonic dystrophy type 1

Linking molecular alterations with disease phenotype

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Abstract

Objective

We aimed to determine whether 3D imaging reconstruction allows identifying molecular: clinical associations in myotonic dystrophy type 1 (DM1).

Methods

We obtained myoblasts from 6 patients with DM1 and 6 controls. We measured cytosine-thymine-guanine (CTG) expansion and detected RNA foci and muscleblind like 1 (MBNL1) through 3D reconstruction. We studied dystrophia myotonica protein kinase (DMPK) expression and splicing alterations of MBNL1, insulin receptor, and sarcoplasmic reticulum Ca(2+)-ATPase 1.

Results

Three-dimensional analysis showed that RNA foci (nuclear and/or cytoplasmic) were present in 45%–100% of DM1-derived myoblasts we studied (range: 0–6 foci per cell). RNA foci represented <0.6% of the total myoblast nuclear volume. CTG expansion size was associated with the number of RNA foci per myoblast ($r = 0.876$ [95% confidence interval 0.222–0.986]) as well as with the number of cytoplasmic RNA foci ($r = 0.943$ [0.559–0.994]). Although MBNL1 colocalized with RNA foci in all DM1 myoblast cell lines, colocalization only accounted for 1% of total MBNL1 expression, with the absence of DM1 alternative splicing patterns. The number of RNA foci was associated with DMPK expression ($r = 0.967$ [0.079–0.999]). On the other hand, the number of cytoplasmic RNA foci was correlated with the age at disease onset ($r = -0.818$ [−0.979 to 0.019]).

Conclusions

CTG expansion size modulates RNA foci number in myoblasts derived from patients with DM1. MBNL1 sequestration plays only a minor role in the pathobiology of the disease in these cells. Higher number of cytoplasmic RNA foci is related to an early onset of the disease, a finding that should be corroborated in future studies.

MORE ONLINE

Video

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Glossary

CI = confidence interval; **DM1** = myotonic dystrophy type 1; **DMPK** = dystrophia myotonica protein kinase; **IR** = insulin receptor; **MBNL1** = muscleblind like 1; **mRS** = modified Rankin Scale; **qPCR** = quantitative PCR; **RT-PCR** = reverse transcription PCR.

Myotonic dystrophy type I (DM1) is a multisystemic disorder with autosomal dominant inheritance caused by a cytosine-thymine-guanine (CTG) repeat expansion in the 3′UTR of the dystrophia myotonica protein (*DMPK*) gene.[1] The main pathogenic process underlying DM1 is a toxic RNA gain-of-function effect of expanded DMPK transcripts forming hairpin-structured aggregates, called "RNA foci."[2,3] These aggregates are able to sequester splicing factors, thereby affecting the alternative splicing of some pre-mRNAs such as insulin receptor (IR), sarcoplasmic/endoplasmic reticulum Ca(2+)-ATPase 1 (ATP2A1),[4,5] or muscleblind like 1 (MBNL1).[6]

Most studies on DM1 have relied on 2D imaging at a single cell level. Yet, this classic approach does not allow detection of all RNA foci aggregates nor identification of their location inside cells. In addition, with this technique, it is not possible to identify the distribution and sequestration of MBNL1 protein. These limitations have hindered recognition of the full cascade of molecular events associated with the phenotype manifestation of DM1.

Here, we used 3D imaging in whole myoblasts derived from patients with DM1 with different degrees of affectation in an attempt to gain insight into the link between DM1 molecular players and disease phenotype. Thus, we assessed the relationship between CTG repeat expansion size, RNA foci, MBNL1 colocalization, and splicing defects in DM1 patient-derived myoblasts, on the one hand, and the clinical characteristics of the patients, on the other hand. We have obtained new pathophysiology data related to how DM1 might affect patient-derived myoblasts.

Methods

Standard protocol approvals, registrations, and patient consents

This study was approved (reference # PI-15-009) by the Ethic Committee of the University Hospital Germans Trias i Pujol (Badalona, Spain) and was performed in agreement with the Declaration of Helsinki for Human Research. All participants signed a written informed consent to participate in the study.

Participants

Six patients were recruited for this study, with DM1 diagnosis confirmed in our laboratory by bidirectional triplet PCR, as previously described.[7] Patient muscle biopsies were obtained from the left biceps brachialis (n = 5) and vastus lateralis muscle (n = 1). The control biopsies were obtained from the hand muscle abductor pollicis longus of healthy individuals (see supplemental data, links.lww.com/NXG/A291, for more information on the participants and clinical data collection).

Myoblast cultures

We extracted muscle cells from the biopsy explants and placed them on culture plates between 2015 and 2016 in the cell culture room of our institution (see supplemental data, links.lww.com/NXG/A291, for more information) and analyzed data from these myoblasts during 2018 and 2019 as described below.

Expansion repeat sizing in myoblasts

We determined CTG expansion size using long PCR-southern blotting (see supplemental data, links.lww.com/NXG/A291, for a detailed description of the protocol).

Three-dimensional imaging of RNA foci and MBNL1

To detect RNA foci and MBNL1, we performed fluorescence in situ hybridization and immunostaining. The protocol we used and the details of 3D imaging can be found in supplemental data, links.lww.com/NXG/A291.

Alternative splicing analysis and *DMPK* expression

We analyzed alternative splicing and *DMPK* expression with reverse transcription (RT) PCR and quantitative PCR (qPCR), respectively (see supplemental data, links.lww.com/NXG/A291, for more information on the protocol and primers).

Statistical analysis

Details of the statistical analysis can be found in supplemental data, links.lww.com/NXG/A291.

Data availability

The data that support the findings of this study are available from the corresponding author on reasonable request.

Results

Patients' phenotype

We studied 6 patients with DM1—4 women and 2 men—with age at disease onset ranging from 15 to 48 years (table 1). Patients were unrelated except P3 and P4 (who were sisters). The majority of patients had cardiac problems, with 3 showing mild ECG alterations, one (P2) a structural cardiopathy (valvulopathy), and another one (P5) using a pacemaker. Half of the patients needed nocturnal mechanical ventilation, and the other 3 had mild changes in respiratory functional tests without actual clinical impairment. The muscular impairment status according to the Muscular Impairment Rating Scale was

Table 1 Clinical data of patients with DM1

Patient with DM1	Sex	Age at onset (y)	Biceps MRC	Myotonia (s)	Cataracts	Cardiopathy	Spirometry	6MWD (m)	MIRS	mRS
P1	F	15[a]	4	0.52	No	LAFB	Altered PFT	348	4	2
P2	M	48	5	0.67	Yes	Valvulopathy	NMV	251	3	2
P3	F	36	5	0.73	Yes	None	NMV	368	2	1
P4	F	42	5	0.98	Yes	1st-degree AV block	NMV	338	3	1
P5	F	27	4	NP	Yes	Pacemaker	Altered PFT	NP	4	4
P6	M	36	5	0.96	No	LAFB	Altered PFT	519	3	2

Abbreviations: 6MWD = 6-minute walking distance; AV = atrioventricular; NP = not performed; LAFB = left anterior fascicular block; mRS = modified Rankin Scale; MIRS = Muscular Impairment Rating Scale; MRC = Medical Research Council Grade; NMV = nocturnal mechanical ventilation; PFT = pulmonary function tests.
[a] It was not possible to determine the actual age at onset of patient P1, but since at the first visit (age 36 years), she had obvious signs that commonly appear in the early patient's life—including oval pallor and temporal atrophy—we considered that the disease onset occurred during adolescence.

variable, with P3 showing minimal signs of muscular impairment on exploration, P2, P4, and P6 presenting with distal weakness, and P1 and P5 with mild-to-moderate proximal weakness. Most patients were independent in activities of daily living (modified Rankin Scale [mRS] score 0–2), yet one (P5) had severely limitations (mRS score 4). P5 did not to do the 6-minute walking distance test or the myotonic evaluation because she died unexpectedly due to respiratory insufficiency at age 40 years.

CTG expansion correlates with RNA foci

We determine the CTG expansion size and the RNA foci and MBNL1 staining analyses in the same cells by dividing the cell lines in different pools. The CTG expansion size in DM1 myoblast cell lines ranged from 195 to 1568 CTG repeats (table 2).

For the staining of RNA foci and MBNL1, we studied 71 and 84 myoblasts from patients and controls, respectively. There was a high heterogeneity among DM1 myoblasts with regard to number of RNA foci. Thus, there was an average of 3 RNA foci (cytoplasmic and/or nuclear) per cell (range 0–6). On the other hand, there were no RNA foci in the control myoblasts (supplemental figure 1, links.lww.com/NXG/A298).

Heterogeneity was also found for the percentage of cells carrying RNA foci. Thus, 4 patients had RNA foci in 100% of their myoblasts, whereas P2 and P6 had RNA foci in 80% and 45% of myoblasts, respectively. Of interest, the cell lines from P2 and P6 were those carrying the lowest CTG repeat number (195 and 230, respectively). We found a strong positive correlation between CTG repeat number and (1) the average number of total (cytoplasmic + nuclear) RNA foci per myoblast (figure 1A), as well as with (2) the average number of nuclear RNA foci per myoblast (r = 0.830; 95% confidence interval [CI] 0.056–0.981).

RNA foci in DM1 myoblasts are mostly, but not only, intranuclear

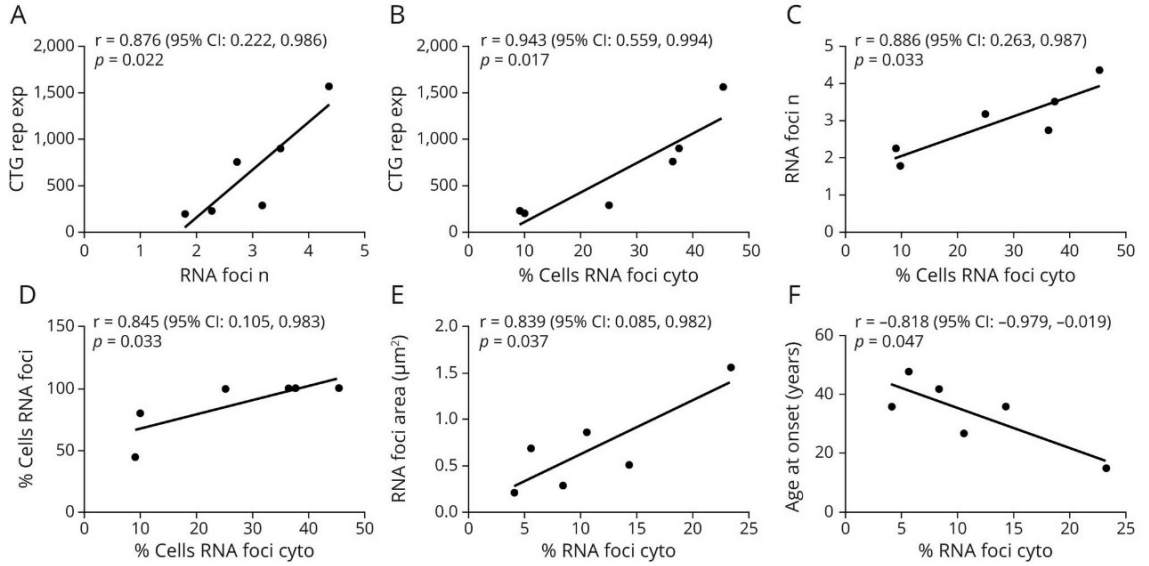
RNA foci were mainly present in the nuclei of DM1 cells. Nevertheless, all 6 DM1 myoblast lines also showed RNA foci outside this organelle. The prevalence of cytoplasmic RNA

Table 2 CTG expansion size and 3D analysis data in DM1 myoblasts

Derived DM1 cell line	CTG repeats	No. of cells analyzed	Minimum no. of RNA foci/cell	Maximum no. of RNA foci/cell	RNA foci/cell	RNA foci area average (μm^2)	% of cells containing RNA foci	% RNA foci in the cytoplasm	% of cells containing both nuclear and cytoplasmic RNA foci
P1	758	11	1	6	2.7	1.6	100	23	36
P2	195	10	0	5	1.8	0.7	80	6	10
P3	899	16	2	6	3.5	0.5	100	14	38
P4	1,568	11	2	6	4.4	0.3	100	8	45
P5	292	12	1	6	3.2	0.9	100	11	25
P6	230	11	0	5	2.3	0.2	45	4	9

Abbreviation: DM1 = myotonic dystrophy type 1.

Figure 1 Significant correlations of 3D analysis data, CTG expansion size, and phenotype in DM1 myoblasts



(A) Correlation between CTG repeat expansion and RNA foci number. The 95% CI for the Pearson correlation coefficient was 0.222–0.986. (B) Correlation between CTG repeat expansion and percentage of cells presenting cytoplasmic RNA foci. The 95% CI for the Pearson correlation coefficient was 0.559–0.994. (C) Correlation between RNA foci number and % of cells presenting cytoplasmic RNA foci. The 95% CI for the Pearson correlation coefficient was 0.263–0.987. (D) Correlation between % of cells presenting RNA foci and % of cells presenting cytoplasmic RNA foci. The 95% CI for the Pearson correlation coefficient was 0.105–0.983. (E) Correlation between the RNA foci area (μm^2) and the presence of cytoplasmic RNA foci. The 95% CI for the Pearson correlation coefficient was 0.085–0.982. (F) Correlation between age at onset of the disease and the presence of cytoplasmic RNA foci. The 95% CI for the Pearson correlation coefficient was −0.979 to −0.019. rep exp = repeat expansion; n = number; cyto = cytoplasm.

foci was, however, heterogeneous—ranging from 4% to 23% of all RNA foci found (mean = 12%) (table 2). The percentage of myoblasts showing cytoplasmic RNA foci also varied among the different DM1 patient lines, ranging from 10% to 45% of cells. On the other hand, there were no cells showing cytoplasmic-only RNA foci.

We found a strong, positive correlation between the number of CTG repeats and the percentage of cells with cytoplasmic RNA foci (figure 1B). We also found a positive correlation between the average total number (nuclear + cytoplasmic) of RNA foci per myoblast and the percentage of cells presenting RNA foci in the cytoplasm (figure 1C) and also between the percentage of DM1 myoblasts presenting RNA foci (nuclear and/or cytoplasmic) and the percentage of RNA foci in the cytoplasm (figure 1D). We further analyzed the area occupied by RNA foci (table 2) and found a positive correlation between this variable and the presence of cytoplasmic foci (figure 1E). Thus, those cell lines that contained RNA foci with larger areas had more foci in the cytoplasm.

Higher number of cytoplasmic RNA foci is associated with an earlier disease onset

When analyzing the relationship between the 3D molecular findings and the phenotype of patients with DM1, we found that the number of cytoplasmic RNA foci was inversely related to the age at disease onset (figure 1F).

RNA foci only represent a small fraction of the total nucleus volume

The portion of the total nuclear dimension occupied by RNA foci was analyzed by determining the volume of all the RNA foci inside the nucleus and by comparing this variable with the total nuclear volume. RNA foci occupied 0.34%–0.53% of the total nuclear volume in DM1 myoblasts.

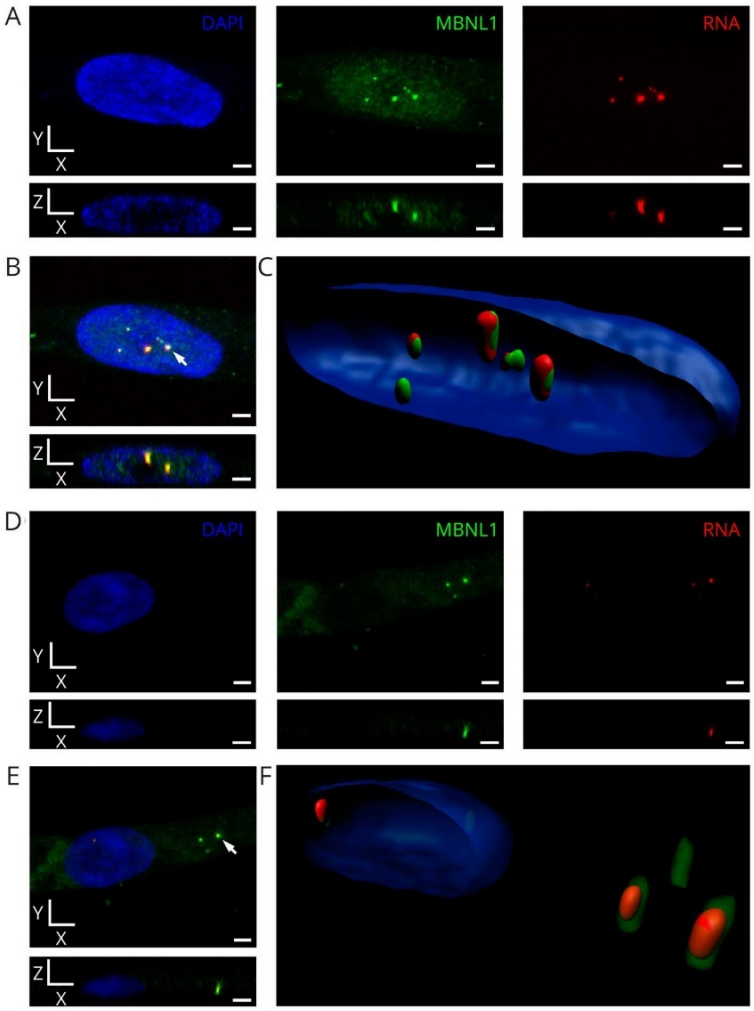
Trapped MBNL1 in RNA foci represent a negligible fraction of total MBNL1 expression

Given the main role that MBNL1 plays in DM1 disease, we aimed to analyze the trapping of this protein in the RNA foci. MBNL1 protein was present in control and DM1 myoblasts. In all analyzed myoblasts, MBNL1 was located both in the nucleus and in the cytoplasm (figure 2, B and E). Colocalization of RNA foci together with MBNL1 was found in all DM1 patients' lines. The trapped MBNL1 was not only found in the nucleus (figure 2, A–C), but also in the cytoplasm (figure 2, D–F), representing 9% of the total cellular colocalization (Video 1). Trapped MBNL1 represented less than 1% of the total MBNL1 expression in DM1 myoblasts.

RNA foci are associated with DMPK transcripts

As RNA foci are the product of *DMPK* gene transcription, we aimed to analyze DMPK transcripts in our myoblast cell lines. To this end, we chose qPCR as it is a more sensitive technique than RT-PCR and used a probe that was located outside the

Figure 2 Confocal microscopy of DM1 myoblasts



MBNL1 protein (green), RNA foci (red), nucleus (blue). Scale bars: 2.5 μm. (A) XY and XZ volume slices of individual channels corresponding to nucleus (blue), MBNL1 protein (green), and RNA foci (red). DM1 myoblast showing the nuclear colocalization between the RNA foci and the MBNL1 protein. (B) Merged image of XY and XZ volume slices of representative nuclear colocalization between RNA foci and MBNL1 protein in DM1 myoblast. White arrow indicates a single RNA foci aggregate colocalizing with MBNL1 protein. (C) Isosurface rendering of nuclear colocalization between RNA foci and MBNL1 protein in DM1 myoblasts. (D) XY and XZ volume slices of individual channels corresponding to nucleus (blue), MBNL1 protein (green), and RNA foci (red). DM1 myoblast showing the cytoplasmic colocalization between the RNA foci and the MBNL1 protein. (E) Merged image of XY and XZ volume slices of representative cytoplasmic colocalization between RNA foci and MBNL1 protein in DM1 myoblast. White arrow indicates a single RNA foci aggregate colocalizing with MBNL1 protein. (F) Isosurface rendering of cytoplasmic colocalization between RNA foci and MBNL1 protein in DM1 myoblasts. MBNL1 = muscleblind like 1.

CTG repeat for detecting expression of both wild-type and expanded *DMPK* alleles. We did not find significant differences between the *DMPK* expression of patients and controls myoblasts (figure 3A). In turn, we found a positive correlation between *DMPK* expression and (1) total (nuclear and/or cytoplasmic) RNA foci number (r = 0.967 [95% CI 0.079–0.999], p = 0.033), as well as with the RNA foci that were present in the nucleus only (r = 0.993 [95% CI 0.682–0.999], p = 0.008).

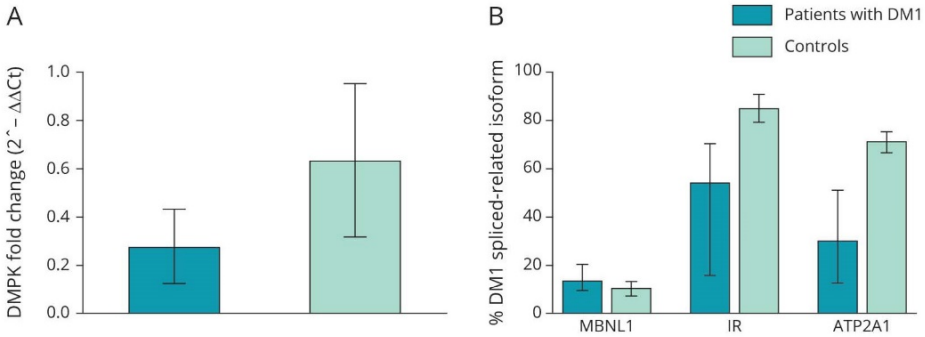
Absence of DM1 alternative splicing patterns in DM1 myoblasts

To study the extent to which was alternative splicing affected in DM1 myoblasts, we analyzed the expression of inclusion and exclusion isoforms—MBNL1 exon 7, INSR exon 11, and ATP2A1 exon 22—that have been previously reported to be altered in DM1.[4,8,9] No differences were found between controls and patients in the expression of MBNL1 or ATP2A1 isoforms. However, the expression levels of the INSR exon 11 exclusion isoform—the most expressed isoform in some DM1 models—showed a tendency of higher expression in controls than in patients (figure 3B).

Discussion

We studied the quantity and distribution of RNA foci and MBNL1 protein in DM1 patient–derived myoblasts using a novel 3D imaging approach. In addition, in the same myoblast cultures, we measured CTG expansion, DMPK expression, and some alternative splicing events. All the molecular data were correlated with the clinical characteristics of the patients with DM1. Thus, although more research is needed, the 3D

Figure 3 qDMPK expression and splicing analysis



Data are mean ± SD. (A) qDMPK expression in patients with DM1 vs controls. (B) Percentage of the DM1 spliced-related isoform in patients with DM1 compared with controls for MBNL1, IR, and ATP2A1 transcripts measured with RT-PCR. MBNL1 and ATP2A1 showed no significant differences, whereas IR showed a tendency of higher expression in controls compared with patients ($p = 0.057$; effect size [Cohen d] = 1.83). DMPK = dystrophia myotonica protein; IR = insulin receptor; MNBL1 = muscleblind like 1.

method we used appears as a potential tool to identify potential associations between molecular alterations and clinical manifestations in patients with DM1.

Our results indicate that the number of RNA foci per DM1 myoblast is regulated by the CTG expansion length. A previous study showed that RNA foci number differed between patients with DM1 carrying <500 or >1000 CTG repeats, respectively.[10] In this regard, we have analyzed patients carrying CTG expansions within the 500–1,000 range, with RNA foci related to CTG repeat number. On the other hand, our data indicate that there are a small number of RNA foci per DM1 myoblast, averaging only 3 per cell (range 0–6), which is consistent with the findings of previous research in muscle tissue.[3,10–12]

Of interest, not all the DM1 myoblasts derived from the same patient had RNA foci. Two lines of patient-derived myoblasts who carried the shorter expansions showed RNA foci only in 40% and 80% of the cells. Thus, our results suggest that CTG expansion not only regulates the amount of RNA foci per myoblast but also the number of myoblasts presenting RNA foci. In this regard, previous research has suggested that RNA foci can undergo dynamic changes during the cell cycle[13] and travel from one cell to another.[14] Thus, although we cannot discard a certain effect of the cell cycle phase on our results, we tried to minimize this potential confounder by seeding the different DM1 myoblast cell lines following a well-defined protocol and using consistently the same treatment method. Based on the expansion sizes that we studied, DM1 myoblasts carrying expansions close to—or larger than—300 CTG repeats would carry RNA foci in all their cells. To study whether 300 CTG repeats is an actual threshold for all patients with DM1 will require a challenging 3D study in muscle tissue of patients with DM1.

We found both nuclear and cytoplasmic RNA foci. Although we found myoblasts with cytoplasmic RNA foci in all the DM1 cell lines that we studied, no myoblast presented cytoplasmic-only RNA foci. CTG expansion size appears to regulate the formation of cytoplasmic RNA foci. Thus, larger CTG expansions will produce a higher number of nuclear foci, and therefore, more cells will contain cytoplasmic foci because the latter only appeared in association with nuclear foci. The presence and origin of cytoplasmic foci have not yet been studied in depth. Some authors have suggested that cytoplasmic RNA foci are a product of the cell cycle as well as of the breakdown of the nuclear membrane and as such are more likely to appear in dividing cells.[13] However, a study in an animal model of DM1 expressing CTG expansions in the heart tissue found RNA foci exclusively in the cytoplasm of cardiomyocytes, which are highly differentiated, nondividing cells.[15] On the other hand, our results show that cytoplasmic RNA foci colocalize with MBNL1. The role of cytoplasmic foci and whether they have a toxic effect should be studied in future research.

RNA foci represent less than 0.6% of the total nuclear volume in DM1 myoblasts. Although researchers in the DM1 area are aware of the small size of RNA foci and of the difficulty to identify them under the microscope by naked eye, the data presented here add some information on the impact that RNA foci have on the nuclear volume. In addition, when analyzing the area of RNA foci in relation to other molecular findings, we observed a correlation with the number of cytoplasmic foci. The fact that scarce data are currently available on cytoplasmic RNA foci makes it difficult to interpret our results. However, as previously described by other authors,[16] RNA foci can fuse or divide randomly with no apparent or known purpose.

Our analysis of MBNL1 revealed that the sequestration of this protein inside RNA foci represents less than 1% of the total MBNL1 myoblast expression. Although there are studies showing the pathologic role of MBNL1 in DM1,[17–21] our

results are consistent with a previous study by Coleman et al.[22] reporting that only a small proportion of MBNL1 protein (0.2%) was sequestered by RNA foci in DM1 lens epithelial cells. The study by Coleman and coworkers also showed that MBNL1 can freely travel from the nucleus to the cytoplasm or from the cytoplasm to the nucleus depending on the transcriptional levels and cell requirements. In this context, the low values of sequestration of MBNL1 would suggest that this phenomenon plays only a minor role in the pathophysiology of the disease, as 99% of total MBNL1 expression is free and DM1 myoblasts showed a distribution pattern similar to that of control myoblasts.

The levels of *DMPK* expression were related to the number of RNA foci per cell. This was an expected finding: indeed, if there are more RNA foci inside a cell, theoretically there should also be more DMPK, as for both RNA foci and DMPK determination, we used a technique, qPCR, which allows detecting expanded as well as wild-type alleles. How *DMPK* expression is affected in DM1 remains to be determined. *DMPK* expression levels have been analyzed in several studies with inconclusive or contradictory results. Some studies found that DMPK transcripts were increased in myotubes derived from patients with DM1,[23] but other authors found the opposite result.[10,24] In this regard, heterogeneity among studies with regard to assessment techniques and patient characteristics limits the ability to gain insight into the alteration of *DMPK* expression in DM1.

We did not find the expected altered DM1 splicing when analyzing IR, ATP2A1, and MBNL1 transcripts. As mentioned above, the trapped MBNL1 in RNA foci was a minor event in DM1 myoblasts, and thus, the cascade of molecular pathomechanisms in which the splicing alteration occurs due to MBNL1 protein sequestration does not seem to play a relevant role. These results could also be explained by the fact that the expression of the aforementioned aberrant isoforms is related to developmental stages,[4,9] and because we used undifferentiated cells (i.e., myoblasts), the expression pattern should differ from the pattern of fully differentiated myotubes, and especially of skeletal muscle fibers.

Our results indicate that an earlier age at disease onset is associated with a higher number of cytoplasmic RNA foci. By contrast, the age at onset was not correlated with the number of CTG repeats or RNA foci per myoblast or to *DMPK* expression levels. Several studies[25–28] have analyzed the possible association between CTG expansion size and the different DM1-related symptoms, but CTG instability adds heterogeneity to the disease, which further complicates to establish these associations.

Our study is limited by the low number of myoblast cell lines studied and by the complexity of 3D molecular imaging at the single cell level, which is a highly time-consuming approach. While keeping this limitation in mind, we were able to evaluate the molecular pathologic cascade in 6 patients with DM1 with different clinical manifestations of this disease.

Our 3D imaging study allowed us to analyze the role of potential DM1 players using a novel approach. Thus, our results would indicate that CTG expansion determines the number of RNA foci per myoblast and of myoblasts that contain RNA foci, as well as the appearance of cytoplasmic foci. An additional finding is that RNA foci only represent a small—and in fact negligible—part of the total nucleus volume and the sequestration of MBNL1 is an infrequent event. Thus, no DM1 splicing alterations were found. The *DMPK* expression levels are related to the number of RNA foci found per myoblast. Last, we found that cytoplasmic RNA foci are inversely related to the age at onset of the patients with DM1 studied. Cytoplasmic RNA foci should be considered in future studies because the role of these molecules in the pathobiology of DM1 needs to be clearly elucidated.

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Disclosure

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Judit Núñez-Manchón, MSc	Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Badalona, Spain	Data acquisition and drafting of the manuscript
Emma Koehorst, MSc	Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Badalona, Spain	Revision of the manuscript
Ian Linares-Pardo	Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Badalona, Spain	Collecting and acquisition of the data
Miriam Almendrote, MD	Hospital Universitari Germans Trias i Pujol, Badalona, Barcelona, Spain	Collecting the clinical data and revision of the manuscript
Giuseppe Lucente, MD	Hospital Universitari Germans Trias i Pujol, Badalona, Barcelona, Spain	Revision of the manuscript
Nicolau Guanyabens, MD	Hospital Universitari Germans Trias i Pujol, Badalona, Barcelona, Spain	Collecting the clinical data
Marta Lopez-Osias, BSc	Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Badalona, Spain	Data acquisition
Adrián Suárez-Mesa, MSc	Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Badalona, Spain	Data acquisition and revision of the manuscript
Shaliza Ann Hanick, MSc	Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Badalona, Spain	Data acquisition and revision of the manuscript
Jakub Chojnacki, PhD	IrsiCaixa AIDS Research Institute, Badalona, Spain	Image analysis
Alejandro Lucia, MD, PhD	Universidad Europea de Madrid and Instituto de Investigación Hospital 12 de Octubre (i+12), Madrid, Spain	Analysis of the data and revision of the manuscript
Guillem Pintos-Morell, MD, PhD	Hospital Universitari Vall d'Hebron, Barcelona, Spain	Revision of the manuscript
Jaume Coll-Cantí, MD, PhD	Hospital Universitari Germans Trias i Pujol, Badalona, Barcelona, Spain	Revision of the manuscript
Alicia Martínez-Piñeiro, MD	Hospital Universitari Germans Trias i Pujol, Badalona, Barcelona, Spain	Revision of the manuscript
Alba Ramos-Fransi, MD, PhD	Hospital Universitari Germans Trias i Pujol, Badalona, Barcelona, Spain	Clinical data collection and drafting of the manuscript
Gisela Nogales-Gadea, PhD	Institut d'Investigació en Ciències de la Salut Germans Trias i Pujol, Badalona, Spain	Design of the study and drafting of the manuscript

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SUPPLEMENTAL DATA: MATERIALS AND METHODS

Participants

Six patients and six controls were recruited for this study. The sample size was limited due to both the very low prevalence of this disorder and the small number of patients who agreed to participate in this study. The patient inclusion criteria were to have confirmed DM1 diagnosis and a complete medical history and follow-up in the neurology department of the aforementioned hospital. Eligibility criteria for the six controls recruited for this study were to have no personal or family history of neuromuscular diseases and to be free of any other chronic disease. Patient muscle biopsies were obtained from the left *Biceps brachialis* (n = 5) and *Vastus lateralis* muscle (n = 1) in the neurology unit or in the ambulatory surgery rooms of our hospital during 2015 and 2016. Controls were healthy individuals – recruited from the Traumatology department, where they had previously undergone minor surgery. The control biopsies were obtained from the hand muscle *Abductor pollicis longus*.

Clinical data

We obtained – and updated – clinical information of DM1 patients from in the last visit's medical records. We also reviewed the last ophthalmological, cardiological and respiratory examination by the corresponding specialists, including the electrocardiograms, echocardiograms and spirometry tests performed in the last year. We assessed the strength of the muscle – *Biceps brachialis* – used for the biopsy in the majority of patients (5 of 6) with the manual Medical Research Council (MRC) scale. We also assessed myotonia, by quantifying the relaxation time – defined as the time from the moment a muscle stops contracting until it relaxes completely, using a new method. In brief, we used an elastic goniometer to calculate the angle of separation between the middle phalanx of the index finger and the metacarpal of the same finger of the dominant hand. Once the goniometer was placed, the patient was asked to perform a maximal contraction in the handgrip (Jammar; Duluth, MN) for 2-4 seconds, and thereafter to relax back to the basal position (hand extension) as fast as possible. This procedure was repeated at least 5 times and an average value of the relaxation time (in seconds) was

obtained. We assessed muscular impairment using the Muscular Impairment Rating scale (MIRS), and evaluated functional status and disability with the 6-minute walking distance (6MWD) test and the modified Rankin Scale (mRS), respectively.

Myoblast cultures

We isolated muscle cells were from muscle tissue by biopsy explants on culture plates treated with human plasma and gelatin 1.5% (1:2). We performed the cultures with DMEM supplemented with 15% of fetal bovine serum, 22% M-199, PSF 1x, insulin 1.74 µM, L-glutamine 2 mM, FGF 1.39 nM and EGF 0.135 mM. We purified myoblasts through CD56 magnetic beads according to manufacturer's instructions (Miltenyi Biotec; Bergisch Gladbach, Germany), and they were further grown on pre-coated surfaces with 0.1% gelatin until 60-70% of confluence was achieved. Myoblasts derived from all DM1 patients and controls were grown simultaneously and plated on coverslips for immune studies. We collected muscle cells in pellets at the same passage of coverslips (from passages 4 to 6), which were frozen at -80°C for further DNA and RNA analysis.

Expansion repeat sizing in myoblast

To measure the expansion size in the myoblast cell lines, we isolated genomic DNA from myoblasts pellets. Briefly, we incubated cell pellets for 16 h at 37°C with 20 mg/mL proteinase K, 1.2 mg/mL Tris-HCl pH 8.0, 0.6 mg/mL NaCl, 7.3 mg/mL EDTA and 10% SDS. The following day, we added 5.5M NaCl before ethanol precipitation. We quantified isolated DNA with Nanodrop ND-1000 (Themo Fisher Scientific; Waltham, MA).

We used long polymerase chain reaction (PCR)-southern blotting to determine the CTG repeat number in cell myoblasts. We amplified genomic DNA from myoblasts in three replicate reactions of 5 ng/µL, 30 ng/µL and 100 ng/µL. We used the primers MDY1D-F GCTCGAAGGGTCCTTGTAGCCG and DM1-rev GTTCCATCCTCCACGCAC. The set conditions of the long PCR were as follows: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s and annealing-extension at 66°C for 8 min. We performed final extension at 65°C for 10 min. We electrophoresed PCR products in a 0.8% agarose gel at 80V for 900 min. We washed the agarose gel with acid

solution (250 mM HCl) for 15 min, basic solution (0.5 M NaOH) for 30 min, and neuter solution (0.5 M Tris-HCl, pH=7.5, 1.5 M NaCl) for 30 min. Thereafter, we transferred the DNA in the gel through capillary electrophoresis to a nylon membrane (Roche, Basel, Switzerland). We fixed DNA to the membrane by heating the latter at 65ºC during 75 min. We used a concentration of 10 pmol/mL DIG-labeled LNA probe (5'-gcAgCagcAgCagCagcAgca-3', where lower and upper-case letters represent unmodified and LNA nucleotides, respectively) to hybridize the membrane for 3 h at 70°C. We detected expansions through chemiluminescence of alkaline phosphatase-conjugated anti-DIG antibody and CDP-Star substrate, following manufacturer's instructions (Roche; Basel, Switzerland). To determine the CTG repeat number, we considered the most abundant band or the most intense part of the smear for diffuse bands, presumably corresponding to the most representative number of repeats present in the myoblasts.

3D imaging of RNA foci and MBNL1

To detect RNA foci and MBNL1, we performed fluorescence in situ hybridization (FISH) and immunostaining. In brief, coverslips containing 60-70% of confluent myoblasts were fixed with 4% PFA and permeabilized with 0.3% Triton X/PBS for 10 min at room temperature (RT). Thereafter we placed them into a humidified chamber and incubated them overnight at 37°C in hybridization buffer: Cy3-$(CAG)_{10}$ probe 0.01 μM, 30% formamide, 10% dextran sulfate, 2mM of vanadyl, 0.02% BSA and 2X SSC. The following day, we washed coverslips three times with 30% formamide/2X SSC for 3 min at 45°C and two times with 1X SSC for three min at 37°C, and did a final wash with 1x PBS for 3 min at RT. Thereafter, we blocked coverslips with 1% goat serum for 1 h in a humidified chamber at RT and thereafter incubated them with the primary anti-MBNL1 antibody mouse 3A4 (sc47740, Santa Cruz Biotechnology; Dallas, TX) 1:100 dilution at 4°C overnight in a humidified chamber. After three washes with PBS, we incubated the cells with secondary antibody anti-mouse Alexa 488 (A11001, Invitrogen by Thermo Fisher Scientific) and diluted then in 1% goat serum/PBS (1/500) for 45 min at RT. After incubation, we washed the cells three times with PBS and mounted them using ProLong Gold antifade reagent with DAPI (Invitrogen; Carlsbad, CA).

We studied colocalization of RNA foci and MBNL1 using a Zeiss LSM 710 confocal microscope (Jena, Germany) equipped with a 63×/1.4 NA oil immersion objective. We

acquired image Z-stacks for each channel sequentially with the following parameters: *i)* pinhole size: 1 Airy; *ii)* XY pixel size: 130 nm; and *iii)* Z pixel size: 300 nm. We analyzed a minimum of 10 myoblasts per cell line and took Z-stack images with an interval of 0.3 µm, covering the total thickness of myoblasts within a 6.3–8 µm range. After their acquisition, we processed the images (cropping and thresholding) using Fiji (ImageJ distribution) software. We generated isosurfaces and 3D video animation using Imaris and Zen Black program, respectively.

Alternative splicing analysis and DMPK expression

We extracted RNA from myoblast pellets using TRIzol™ Reagent (Thermofisher), following the manufacturer's instructions. We treated RNA with *DNAse I* and converted it to cDNA using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher). We analyzed DMPK expression with quantitative PCR (qPCR) using Taqman Fast Advanced Mastermix and the following custom Taqman assay (ThermoFisher): forward AGCCTGAGCCGGGAGATG, reverse GCGTAGTTGACTGGCGAAGTT and probe AGGCCATCCGCACGGACAACC (2). We analyzed the results with the 2-ΔΔCT method using human large ribosomal protein (RPLPO) as endogenous control. We analyzed alternative splicing of MBNL1, INSR and ATP2A1 with reverse transcript (RT)-PCR. For RT-PCR analysis, we kept annealing temperature at 58ºC for 50 cycles and used the following primers: MBNL1 forward GCCCAATACCAGGTCAACCA (3), MBNL1 reverse GGCCTCTTTGGTAATGGGGG (3), INSR forward GAGCTGGAGGAGTCCTCGTTTAG, INRS reverse TCGATGCGATAGCCCGTGAAG, ATP2A1 forward CTCATGGTCCTCAAGATCTCAC (4) and ATP2A1 reverse AGCTCTGCCTGAAGATGTGTCAC (4). We tested the amplified products in a 3% high-resolution agarose gel and quantified bands with ImageJ. We calculated the percentage of exon inclusion and exclusion isoforms as follows:

$$\frac{\text{exon eclusion OR inclusion band}}{(\text{exon exclusion } + \text{ exon inclusion band})} \times 100$$

Statistical analysis

We analyzed the relationship between molecular (3D) and clinical data using Pearson's correlations after checking normality with the Kolmogorov-Smirnov test. We applied the Mann Whitney's *U* test to compare expression and alternative splicing profiles in DM1 vs control myoblasts. We performed all statistical analyses with the Graphpad Prism 5 software, with the level of significance set at 0.05.

Video 1

Video animation of DM1 myoblasts. DM1 myoblasts showing colocalization between RNA foci and MBNL1 protein both in the nucleus and in the cytoplasm. Download Supplementary Video 1 via http://dx.doi.org/10.1212/000484_Video_1

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Chapter V

BNA^{NC} treatment has a different efficacy and toxicity in fibroblasts, lymphoblasts and myoblasts derived from Myotonic Dystrophy Type 1 patients

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Abstract

Myotonic dystrophy type 1 (DM1) is a neuromuscular disease with multisystemic effects and no known cure. Several studies, using antisense oligonucleotides (ASOs) as a potential therapy, have shown promising results, although the required efficacy for proper treatment is hard to reach due to poor delivery and toxicity. BNA^{NC} are an ASO type with specific chemical modifications that have shown to provide an extra efficiency with less toxicity than other ASOs. However, it is not known yet whether once reaching the target tissues, the treatment will be equally effective to the different cell types, and whether the CTG expansion size of DM1 patients is related to the treatment response. We establish through the use of DM1 patient derived fibroblasts that the most effective BNA^{NC} gapmer was the one directed against the CUG repeat at 30 nM after 48h of treatment. We used these conditions to treat three cell types derived from eight DM1 patients (fibroblasts n=8, lymphoblasts n=6, and myoblasts n=6), in which the tissues for deriving those cells, were obtained simultaneously. Our results showed that BNA^{NC} treatment has very heterogenous effects on the DM1 cells, between and within the patients. BNA^{NC} compounds showed different efficacy depending on the cell type reducing the RNA foci in fibroblasts (79%), lymphoblast (24%) and myoblasts (15%). The mortality of the cells due to the treatment is different between the cell types as well, lymphoblasts showed the highest toxicity with 13% of mortality, followed by fibroblasts (8%) and myoblasts (2%). Finally, BNA^{NC} treatment efficacy in terms of RNA foci reduction and the treatment toxicity in terms of cell mortality seems to be independent of the CTG expansion length.

Introduction

Myotonic Dystrophy Type 1 (DM1) is a multisystemic disorder. Symptoms of DM1 include myotonia, atrophy and muscle weakness [1]. However, muscle is not the only affected tissue in DM1 patients, and other symptoms such as cataracts, cognitive impairment, cardiac and respiratory problems, gastrointestinal and endocrinal affectation are also present in these patients [2]. DM1 is a genetic disease with an autosominal dominant inheritance. The mutation underlying DM1 is a CTG repeat expansion in the 3'UTR region of *dystrophia myotonica* protein kinase (*DMPK*) gene [3]. DM1 patients carry from 50 to over thousand repeats [4]. The CTG expansion is highly unstable and results in a somatic mosaicism of different CTG expansion lenghts present in the different tissues [5]. The CTG expansion causes the formation of toxic RNA aggregates that accumulate in the nucleus of the cells [6]. These toxic aggregates, called RNA foci, trap different nuclear proteins such as the splicing factor MBNL1, producing significant splicing alterations that affect the cellular function [7]. Although DM1 is the most common muscle dystrophy in adults, there is no treatment to cure or to stop disease progression, yet.

Antisense Oligonucleotides (ASOs) are molecules which are widely studied as a possible therapeutic approach for several diseases, including DM1. The addition of chemical modifications such as phosphorothioate (PS), 2'-O-methoxyethyl (MOE) or 2'-O,4'-C-methylene-locked nucleic acid (LNA), improves the stability and modifies the pharmacokinetic properties of ASOs [8]. Gapmers are a specific type of ASO that are especially useful to target and degrade the RNA foci, since they activate the endonuclease RNase H1 activity [9,10]. However, 2' ribose modifications are not compatible with RNase H1-mediated target degradation. Thus, gapmer compounds have a special structure containing 2' ribose modifications only in the 5' and 3' ends, which increases ASO binding affinity and nuclease resistance, and a nucleotide "gap" with chemical modifications to maintain the RNase H1 activity [8,10].

Different studies have analyzed the therapeutic potential of different ASOs in the DM1 field, in both transfected cells and animal models [11–13]. In 2017, a clinical trial using a MOE gapmer (IONIS-DMPKRx) demonstrated promising results, although Ionis Pharmaceuticals is currently working on improving tissue delivery [14]. Other studies have compared MOE and LNA compounds, showing that LNA are 5-10 fold more potent [15,16]. However, the high affinity of LNA can also result in toxic effects

due to nonspecific off-target binding, and multiple studies have shown hepatoxicity and apoptosis in both cell and mice models. The 2′-*O*,4′-methylene bridged nucleic acid [NMe] (BNA^{NC}) modifications also include a locked structure similar to the LNA compounds, which increases the affinity of the molecules [17,18]; however, they have shown to be better tolerated in animal models [19]. Moreover, one previous study has tested BNA^{NC} gapmers in DM1 transfected COSM6 cells, showing that they have comparable potency to LNA gapmers in DM1, as well as a lower propensity to induce caspase activity [20]. Therefore, BNA^{NC} gapmer modifications are a promising alternative for the therapeutic development in DM1. However, the efficacy of these molecules to degrade RNA foci in cells derived from patients, has not been evaluated yet. Moreover, being DM1 a multisystemic disease, little is known about whether the treatment with ASOs would equally affect the different tissue of the body, and whether the CTG expansion length is related to the treatment response.

We obtained skin fibroblast, lymphoblast, and myoblast cell lines derived from eight DM1 patients in which the tissues were extracted at the same time. We compared the response of each cell type after 48 h of treatment with a BNA^{NC} gapmer directed against the CUG repeat. Our study shows that ASOs have a different efficacy and toxicity depending on the treated cell type, and that a high heterogeneity exists in the response between and within the treated patients.

Materials and Methods

Participants and sample collection

The present study was aproved by the ethics commmittee of the University Hospital Germans Trias i Pujol, # PI15-009, and was in agreement with the Declaration of Helsinki for Human Research. We obtained the informed consent from all the participants of the present study. Eight DM1 patients were included in the study.

We obtained blood, a skin and a muscle biospy from each patient at the same time. Blood was obtained from peripheral blood extraction. Muscle biopsies were obtained from the left *Biceps brachialis* (n = 7) and *Vastus lateralis* muscle (n = 1), and skin biospies were obtained by a 0.5 cm skin punch. Blood was collected in heparin tubes and peripheral blood mononuclear cells (PBMCs) were isolated using a

standard ficoll procedure. PBMCs were incubated with anti-human CD3 antibody to supress T cells, and subsequently immortalized with Epstein Bar virus. Lymphoblasts where further cultured with B95-8 medium (80% RPMI, 10% Fetal Bovine Serum, PSF 1x and L-Glutamine 200 nM). Muscle and skin biopsies were seeded in plates with human serum and gelatin 1.5% (1:2), and cultured with DMEM supplemented with 5% of Fetal Bovine Serum and PSF 1x, at 37°C for two-three days. We obtained derived cells from the tissue explants that we further cultured with DMEM supplemented with 15% of fetal bovine serum and PSF 1x in the case of the fibroblasts and supplemented as well with 22% M-199, insulin 1.74 µM, L-glutamine 2 mM, FGF 1.39 nM and EGF 0.135 mM was used in the case of the derived muscle cells. We purified myoblasts through CD56 magnetic beads according to manufacturer's instructions (Miltenyi Biotec; Bergisch Gladbach, Germany), and they were further grown on pre-coated surfaces with 0.1% gelatin until 60-70% of confluence was achieved. We could obtain derived fibroblasts cell lines from the eight patients, whereas lymphoblasts and myoblasts could be obtained only from six patient cell lines. Skin fibroblast, lymphoblast and myoblast cell lines were frozen at -80°C for further BNA^{NC} experiments.

DNA extraction and CTG expansion sizing

We defrosted each cell type and we plated the cells spliting into different groups, treated and non-treated cells. To measure the CTG expansion size present in the different cell types, we first extracted the DNA from a cell pellet obtained from the non-treated cells group. We extracted the genomic DNA from the cell pellets after a 16 hours (h) incubation at 37°C with 20 mg/mL proteinase K, 1.2 mg/mL Tris-HCl pH 8.0, 0.6 mg/mL NaCl, 7.3 mg/mL EDTA and 10% SDS. The following day, we added 5.5 M NaCl before ethanol precipitation. We quantified the isolated DNA with Nanodrop ND-1000 (Themo Fisher Scientific; Waltham, MA).

To measure the CTG expansion size, we performed small pool-PCR, described elsewhere [21]. DNA fragments were resolved by electrophoresis on a 1% agarose gel, followed by southern blot [22]. The estimated CTG sizes of the progenitor, the mode and the longest allele were measured for each cell type by comparison against the molecular weight ladder, using GelAnalyzer 19.1 software.

BNA^{NC} treatment

We used three different BNA^{NC} gapmers that were purchased from Bio-Synthesis. The BNA^{NC} compounds that we used have been based on the previous study of Manning and colleages [20], and had the following sequences: AGCagcagcagCAG (directed against the expansion repeat and will be referred to as CAG), CGGAGcggttgtgaaCTGGC (targeting 121 bp upstream of the CUG expansion, and will be referred to as DMPK) and TCGtgctgctgCTG (targeting non specifically an unkown region of the genome, and will be referred to as C), where capitals mean BNA^{NC} modifications. All ASOs were fully phosphorothioated. BNA^{NC} gapmers were delivered to the cells by 1.6% lipofectamine 2000 (Thermo Fisher Scientific) in Opti-Mem medium. We treated fibroblasts with the three compounds (CAG DMPK and C), using the following doses: 10 nM, 30 nM and 60 nM, for 24 h and 48 h, at 37°C. After the evaluation of the results, fibroblasts, lymphoblasts and myoblasts were treated with CAG at 30 nM during 48 h, at 37°C.

FISH and foci quantification

We detected RNA foci by fluorescence in situ hybridization (FISH). Briefly, coverslips containing non-treated and treated fibroblasts, lymphoblasts and myoblasts with 60-70% confluency were fixed with 4% PFA and permeabilized with 0.3% Triton X/PBS for fibroblasts and myoblasts and 0.1% Triton X/PBS for lymphoblasts, for 10 min at room temperature (RT). Thereafter we placed them into a humidified chamber and incubated them overnight at 37°C in hybridization buffer ($Cy3\text{-}(CAG)_{10}$ probe 0.01 µM, 30% formamide, 10% dextran sulfate, 2mM of vanadyl, 0.02% BSA and 2X SSC). The following day, we washed coverslips three times with 30% formamide/2X SSC for 3 min at 45°C and two times with 1X SSC for three min at 37°C, and did a final wash with 1x PBS for 3 min at RT. We mounted the coverslips using ProLong Gold antifade reagent with DAPI (Invitrogen; Carlsbad, CA).

Images were obtained with a Zeiss AxioObserver Z1 microscope (Jena, Germany), using a 63×/1.4 NA oil immersion objective. After image acquisition, we quantified the RNA foci number using Fiji (ImageJ distribution) software. We analyzed the RNA foci/cell in non-treated and treated cells after 24 h and 48 h, in at least 20 arbitrary cells per each condition and each patient cell type, to calculate the reduction of RNA foci/cell.

Cell mortality quantification

We quantified the cell mortality in non-treated and in treated cells after 24 h and 48 h, using a ReadyProbes™ Cell Viability Imaging Kit (Invitrogen by Thermo Fisher Scientific). Cell mortality was analyzed in 200 arbitrary cells for each condition in each patient cell type.

Statistical analysis

We used a one-way analysis of variance (ANOVA) to compare RNA foci reduction and mortality, respectively, among the different cell types. The unpaired Students' t-test was used to compare treated *vs* non-treated cells. The relationship between CTG expansion size and treatment response (*i.e.*, RNA foci reduction and cell mortality), as well as of the treatment responses among the different cell types within the same patient were analyzed using Pearson's and Spearman correlations for normally and non-normally distributed variables, respectively (normality assessed the Shapiro-Wilk test). The level of significance was set at 0.05 (two-tailed).

Results

24 h of treatment was not enough to reduce RNA foci in DM1 skin fibroblasts

To test which were the best treatment conditions, we first compared two treatment timings, 24 h and 48 h of treatment (with CAG, DMPK and C) at 10 nM, 30 nM and 60 nM, in skin fibroblasts of one patient. Our results showed that the efficacy in reducing the RNA foci and the toxicity of the treatment in skin fibroblasts was highly variable depending on the BNA^{NC} compound (CAG, DMPK or C) (**Figure 1**). The reduction of RNA foci after 24 h of treatment was not significant for any of the three compounds. Cell mortality after 24 h of treatment increased slightly, especially when using the highest concentration of each compound (around 10% more cell mortality compared to the non-treated cells).

After 48h of treatment the RNA foci were visually reduced, but only when using the CAG BNA^{NC} compound (from 1.29 RNA foci/cell in non-treated cells to 0.37 RNA

foci/cell in 10 nM CAG, 0.22 RNA foci/cell in 30 nM CAG and 0.17 RNA foci/cell in 60 nM CAG). DMPK and C BNA^{NC} compounds did not show any significant RNA foci reduction after 48 h of treatment. Referring to the treatment toxicity, the cell mortality rose progressively when increasing the compound concentration, arriving to a 99% and 96% of cell mortality in the case of DMPK and C compounds, respectively, when treating at 60 nM dose. However, the increase in the cell mortality when using CAG BNA^{NC} compound was only 22% more than in non-treated cells at 10 nM dose, 10% at 30 nM dose and 40% at 60 nM dose.

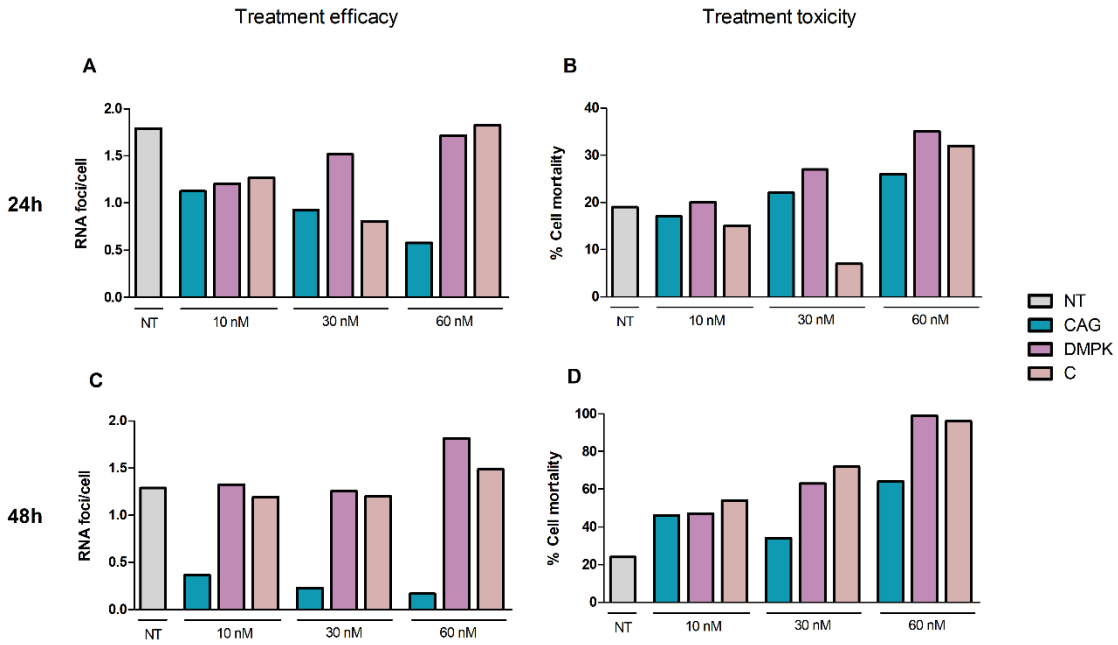

Figure 1. Treatment efficacy and toxicity after 24 and 48 h in DM1 patient's fibroblasts. (**A**) RNA foci reduction (RNA foci number/cell) after 24 h of treatment in DM1 fibroblasts; *n*=1. (**B**) Cell mortality (%) after 24 h of treatment in DM1 fibroblasts; *n*=1. (**C**) RNA foci reduction (RNA foci number/cell) after 48 h of treatment in fibroblasts; *n*=1. (**D**) Cell mortality (%) after 48 h of treatment in DM1 fibroblasts; *n*=1. NT = non-treated cells.

CAG BNA^{NC} compound reduced the RNA foci number without increasing the cell mortality

We repeated the previous experiment in skin fibroblasts of three different patients using the three BNA^{NC} compounds (CAG, DMPK and C), at the three different doses (10 nM, 30 nm and 60 nM), but only during 48 h of treatment (**Figure 2**). RNA foci reduction was only significant when using the CAG BNA^{NC} compound at 30 nM (p = 0.018) and 60 nM doses (p = 0.017) (from 1.58 RNA foci/cell in non-treated cells to 0.17 RNA foci/cell and 0.19 RNA foci, respectively). Cell mortality after 48 h was only significant when treating with DMPK (p = 0.020) and C (p = 0.038) at 60 nM (65% and 51%, respectively, of cell mortality increase compared to non-treated cells). By contrast, the cell mortality with CAG BNA^{NC} compound at 30 nM and 60 nM was not significant, only 3% and 25% more than non-treated cells, respectively. After these results we decided that the best treatment conditions to use in the next experiments were CAG BNA^{NC} compound, at 30 nM and during 48 h.

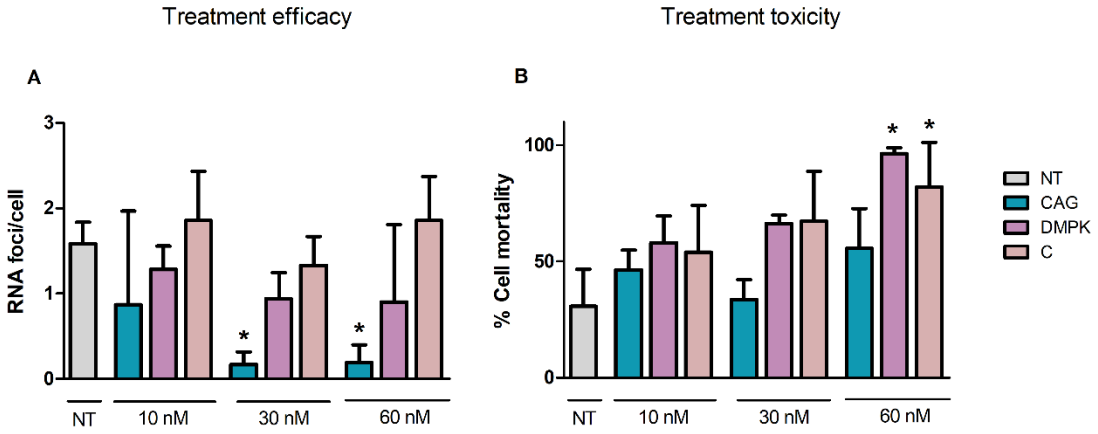

Figure 2. Treatment efficacy and toxicity after 48 h in DM1 patients' fibroblasts. (**A**) RNA foci reduction (RNA foci number/cell) after 48 h of treatment in DM1 fibroblasts; n=3. (**B**) Cell mortality (%) after 48 h of treatment in DM1 fibroblasts; n=3. NT = non-treated cells. * p *value* < 0.05.

RNA foci reduction was different in each cell type

To evaluate the efficacy of the treatment we treated the three cell types (fibroblasts, lymphoblastsand and myoblasts) with the CAG BNA^{NC} compound, at 30 nM and during 48h, and we quantified the RNA foci number afterwards. **Figure 3** shows the presence of the RNA foci aggregates before and after the treatment in the

three different cell types. Our results showed that the reduction of the RNA foci was different in each cell type (p = 0.0002) (**Figure 4**). Fibroblasts were the cell type which showed higher RNA foci/cell reduction (79%), followed by lymphoblasts which showed 24% of reduction and finally myoblasts, which showed 15% of RNA foci/cell reduction. We found differences within each cell type as well, with a RNA foci/cell average reduction from 22% to 100% in fibroblasts, from 0% to 62% in lymphoblasts, and from 0% to 37% in myoblasts.

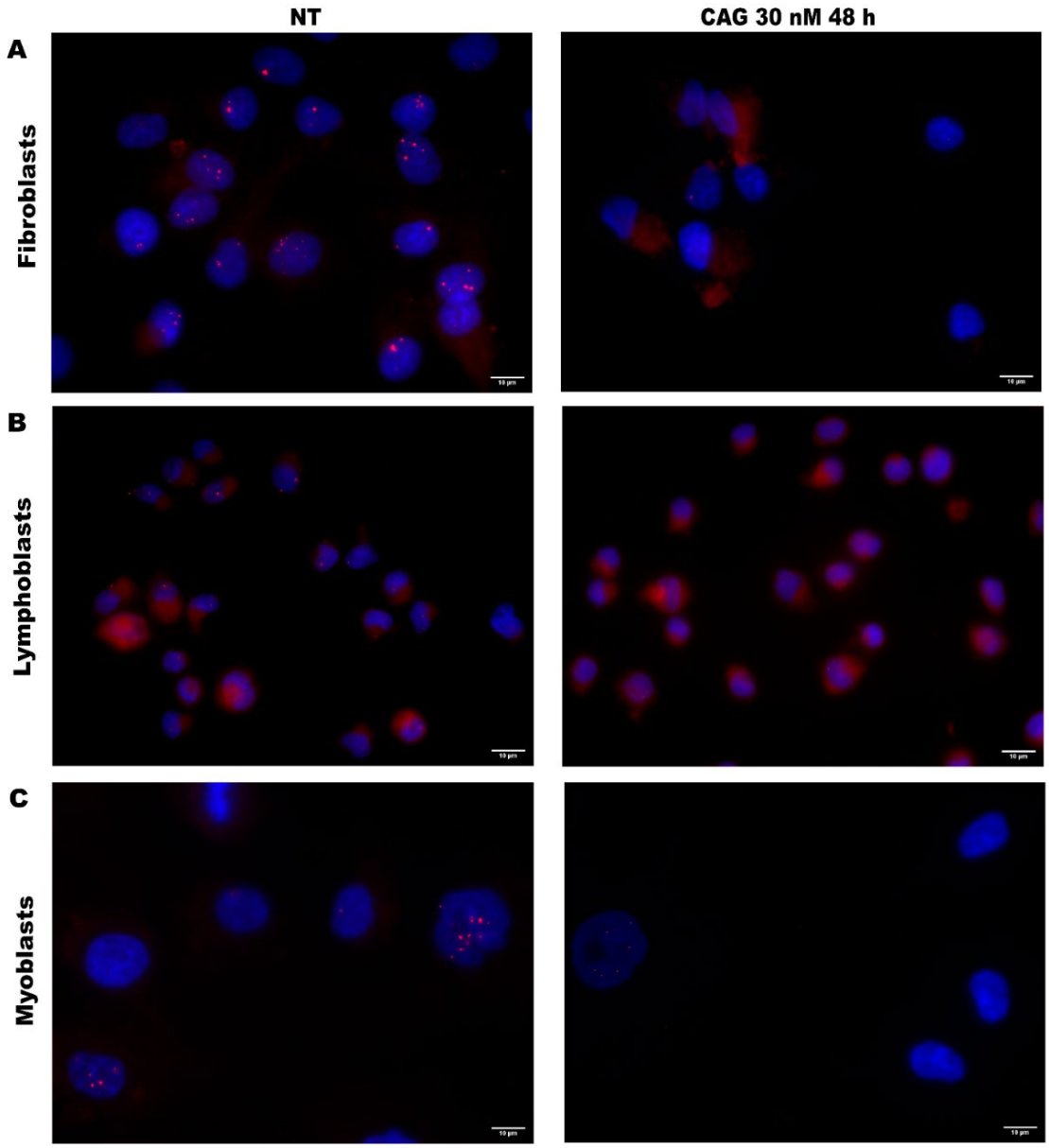

Figure 3. RNA foci reduction in fibroblasts, lymphoblasts and myoblasts. RNA foci (red), cell nucleus (blue). Scale bars: 10 µm. (**A**) RNA foci (red) in fibroblasts before (NT) and after (CAG 30 nM 48 h) the treatment. (**B**) RNA foci (red) in lymphoblasts before (NT) and after (CAG 30 nM 48 h) the treatment. (**C**) RNA foci (red) in myoblasts before (NT) and after (CAG 30 nM 48 h) the treatment. NT = non-treated cells.

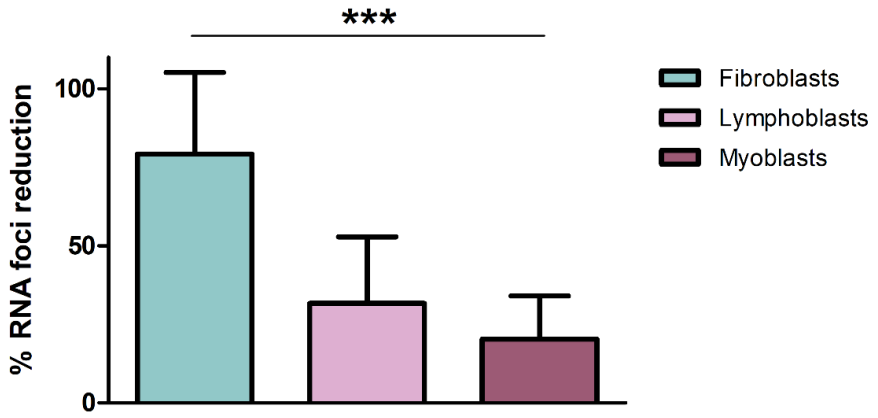

Figure 4. RNA foci reduction (%) in fibroblasts, lymphoblasts and myoblasts after 48 h of CAG 30 nM treatment. % of RNA foci number/cell reduction in fibroblasts (79%), lymphoblasts (24%) and myoblasts (15%). *** *p value* < 0.001.

Each cell type showed different mortality in response to the treatment

We evaluated the cell mortality after the treatment in the different cell types, and each cell type showed different mortality in response to the treatment (**Figure 5**). Lymphoblasts showed higher mortality after the treatment (18%), compared to fibroblasts (8%) and myoblasts (3%) (p = 0.005). However, when comparing with the non-treated cells, the cell mortality after the treatment only increased significantly in fibroblasts (p = 0.023) (5% more than non-treated cells). We also quantified the toxicity that was associated to the lipofectamine itself in every cell type, and we did not find any significant increase in the cell mortaly due to the lipofectamine compared to the non-treated cells.

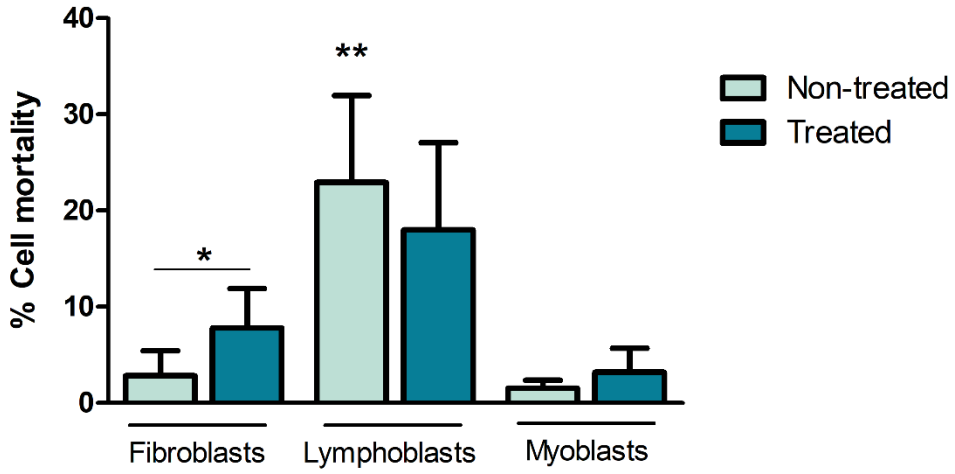

Figure 5. Cell mortality (%) in fibroblasts, lymphoblasts and myoblasts after 48 h of CAG 30 nM treatment. Comparation between cell mortality in non-treated and treated cells (CAG 30 nM 48 h). NT = non-treated cells. * *p value* < 0.05; *** *p value* < 0.001.

CTG expansion size did not correlate with the RNA foci reduction or cell mortality

We wondered whether the different cell types coming from the same patient would show the same RNA foci reduction and cell mortality tendency after the treatment in each cell type. Thus, we analyzed whether the heterogeneity found in the the treatment response of each cell type was patient depended. However, no correlations were found between the cell types coming from the same patient. Furthermore, we also wanted to study whether the treatment response was related to the CTG expansion size present in the cells. Likewise, no significant correlations were found between the CTG expansion length of any cell type and their response to the treatment, not regarding the RNA foci reduction and neither the cell mortality.

Discussion

After testing different BNA^{NC} compounds at different doses and treatment durations, we found that CAG BNA^{NC} compound at 30 nM dose and during 48 h were the best treatment conditions when analyzing the RNA foci reduction and the cell mortality. Thus, we used these conditions to treat three different cell types (fibroblasts, lymphoblasts and myoblasts) derived from DM1 patients and analyze the treatment response in each cellular type.

Our results showed that 24 h of treatment was not enough time to show a significant RNA foci reduction. Manning and colleagues showed that 24 h of CAG BNA^{NC} compound treatment did show a significant knockdown of the mRNA levels in COSM6 transfected cells, even at 0.3 nM dose [20]. This suggests that the efficacy of these therapies in transfected cells may be higher, especially considering the overexpression of the expanded *DMPK* compared to DM1 patient derived cells. Regardless, our results showed a slight increase in the cell mortality after 24 h when using the highest dose of each compound, suggesting that 60 nM was in general a too high of a dose. After 48 h of treatment, we only observed significant RNA foci reduction when treating with the CAG BNA^{NC} compound, at 30 nM and 60 nM, whereas DMPK BNA^{NC} compound did not show any significant RNA foci reduction at any of the tested doses. Interestingly, the study mentioned previously showed that

CAG BNA^{NC} compounds displayed more potent mRNA knockdown than DMPK BNA^{NC} compound at lower doses (30 nM) but DMPK BNA^{NC} compound were more effective than CAG ones at a higher dose (100 nM) [20]. They hypothesize that at low concentrations the CAG BNA^{NC} compound have more binding sites per molecule, and at higher concentrations the CUG repeat is partially blocked, probably by MBNL proteins or RNA structure. However, we found that at the higher dose we used (60 nM), the RNA foci reduction seen after DMPK BNA^{NC} compound treatment was not significant, and moreover, it was related to a significant increase in the cell mortality. Contrary, Manning *et al*. did not find cytotoxic effects using either CAG and DMPK BNA^{NC} compound at 30 nM [20]. The fact that they did not find cytotoxic effects could indicate that patient derived cells are more sensitive to the toxicity of a BNA^{NC} treatment than transfected cells. Moreover, we have treated cells that contain different CTG expansion sizes, from 329 to 1875 CTGs, whereas in the previous study only transfected cells containing 300 CTGs have been studied. In accordance to the hypothesis from Manning *et al.*, this could be related to the fact that when treating cells containing longer CTGs there are more binding sites per molecule, explaining the higher RNA foci reduction seen in CAG BNA^{NC} compound compared to DMPK BNA^{NC} compound, and also the higher dose that is required in patient derived cells to achieve a significant RNA foci reduction compared to transfected cells.

When comparing the treatment efficacy between the cell types, our results showed that the CAG BNA^{NC} compound reduced the RNA foci in fibroblasts, lymphoblasts and myoblasts of DM1 patients. However, the average number of RNA foci reduction was different for each cell type. This suggests that each cell type may respond different due to the cell membrane properties that modify the BNA^{NC} compound efficacy to penetrate into the cells. Experiences in the laboratory indicate that myoblasts are in general difficult to transfect, which could explain the fact that they showed the lowest RNA foci reduction after the treatment. Achieving the lowest efficacy in myoblasts, which are derived from one of the most affected tissues in DM1 patients, represents an issue for the DM1 treatment approach, and it is actually the reason why the ISIS clinical trial was halted, due to the limited muscle uptake. Our results also highlight the necessity of focusing on the improvement of muscle delivery. The fact that lymphoblasts, derived from one of the most accessible tissues, did not show a cell mortality increase due to the treatment, indicates that may be a possibility to increase an intravenous treatment dose in order to reach other tissues with more

efficacy. Moreover, the cell mortality in myoblasts due to the treatment was not significant, which is in accordance to the idea that the delivery into these cells is limited.

Besides the heterogenous treatment response found between the cell types, we also found differences between each patient's cell line. When we studied whether this response was patient-related and whether fibroblasts, lymphoblasts and myoblasts from a same patient had the same response tendency to the treatment, we did not find any correlation, meaning that the response to the treatment is specific to each cell type, independently to the patient. This suggests that although the treatment would be better tolerated and more effective in one patient tissue, another tissue from the same patient could be reacting very differently to the same treatment. Moreover, we also analyzed whether the CTG expansion size was related to the treatment response. It is known that the CTG expansion length is related to the age of disease onset and to the MRC in the muscle [23,24], and thus, it would be interesting to see whether the cells carrying longer expansions, which could be more severely affected, would show the same treatment efficacy using the same BNA^{NC} compound dose compared to other cells carrying shorter expansions. However, we did not find any correlation, suggesting that the treatment response is independent of the CTG expansion size.

We were also interested in analyzing the effects that the treatment had on the sequestration of the MBNL1 protein into the RNA foci. Using CAG BNA^{NC} compound 30 nM 48 h we have seen 100% reduction of the RNA foci only in one patient, meaning that in general lines, the presence of the toxic aggregates does not disappear completely and although the MBNL1 will be probably trapped in a less fraction, we are interested in studying whether in the residual RNA foci the CUG binding sites are blocked by the ASO, and the level of sequestered MBNL1 is even less. Additionally, we were interested in analyzing whether the treatment is also targeting the non-expanded CUG coming from the expression of the wild type *DMPK*. However, the results of the quantitative-PCR quantifying the *DMPK* gene expression are pending to be analyzed.

In conclusion, we have shown that DM1 cells respond differently to a BNA^{NC} treatment. This hypothesizes that when a specific ASO therapeutic approach will be

administered and delivered equally to the tissues, the treatment efficacy and toxicity among tissues and patients will not be the same.

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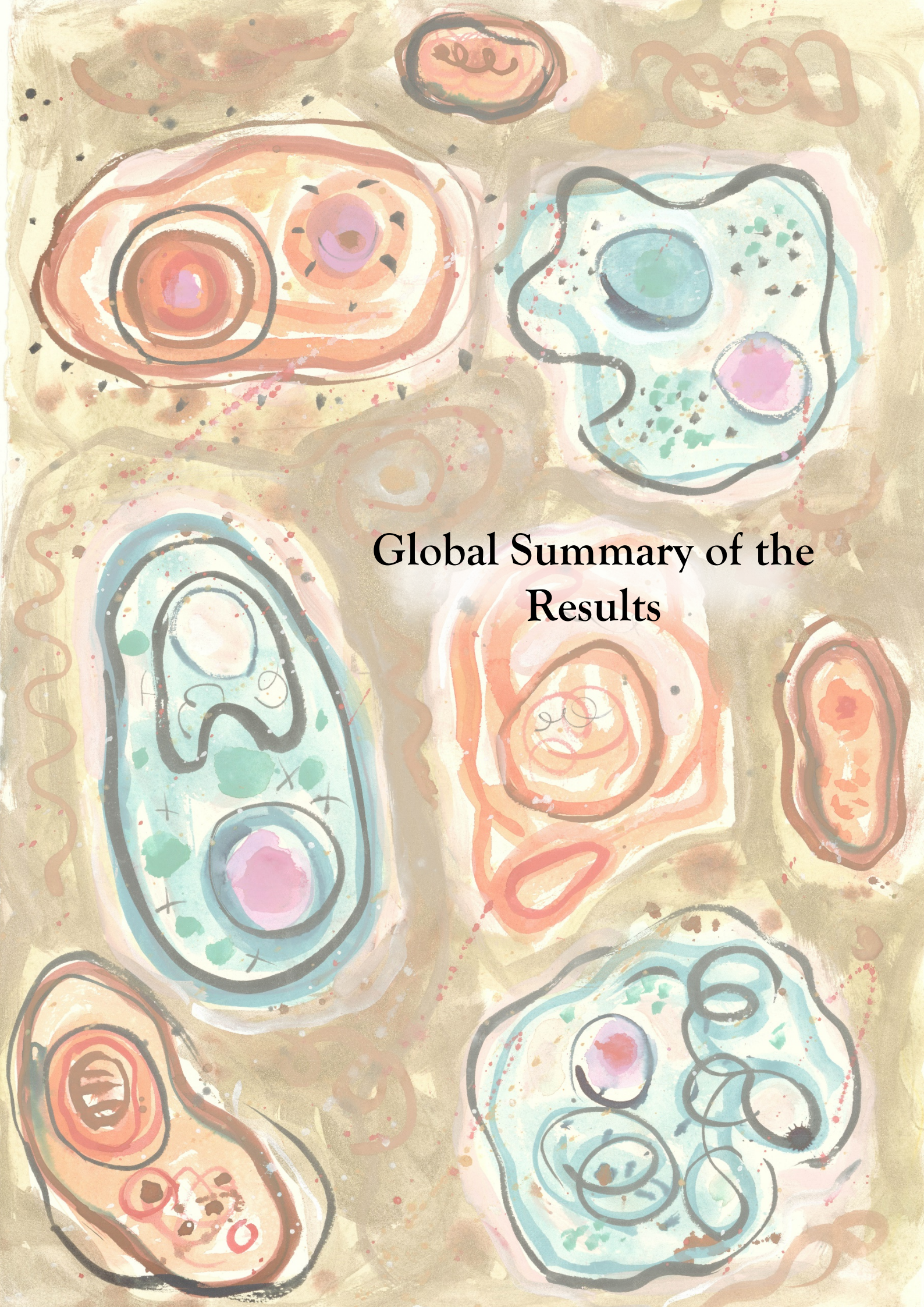
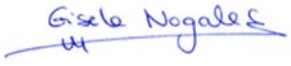


Global Summary of the Results

Report of the supervisor

En la presente tesis de Alfonsina Ballester López están incluidos tres artículos publicados, y un cuarto en revisión, que esperamos que en las próximas semanas sea aceptado para su publicación. El primer artículo aceptado que forma el capítulo III de la tesis es ***A DM1 family with interruptions associated with atypical symptoms and late onset but not with a milder phenotype***. Este artículo se publicó en 2019, en la revista internacional *Human Mutation*, que tiene un factor de impacto de 4,370. En este trabajo Alfonsina Ballester es primera autora firmante. Ha hecho la mayoría de los experimentos, ha analizado sus resultados, y ha participado en la escritura y revisado el contenido final del manuscrito. El segundo artículo aceptado es ***Three-dimensional imaging in myotonic dystrophy type 1: Linking molecular alterations with disease phenotype***. Este artículo forma parte del capítulo IV de la tesis, y se publicó en la revista internacional *Neurology Genetics*, que tiene un factor de impacto en 2019 de 3.65. En este trabajo Alfonsina Ballester también es primera autora firmante. Ha hecho la mayoría de los experimentos, ha analizado sus resultados, y ha escrito el manuscrito y revisado su contenido final. Finalmente, el último artículo aceptado que forma parte de la tesis es ***The Need for Establishing a Universal CTG Sizing Method in Myotonic Dystrophy Type 1.*** El artículo forma el primer capítulo de la tesis y se publicó en la revista internacional *Genes*, que tiene un factor de impacto en 2019 de 3.331. En este trabajo Alfonsina Ballester es primera autora firmante. Igualmente, ella ha hecho la mayoría de los experimentos, ha analizado sus resultados, y ha escrito el manuscrito y revisado el contenido final del manuscrito.

Todos los trabajos anteriormente citados no han sido utilizados para la realización de ninguna otra tesis anteriormente.



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Global Summary of the Results

Regarding the **Chapter I**, in order to determine whether different sizing techniques yielded the same CTG expansion size in DM1 patients, we compared the results obtained from 15 DM1 patients' blood using three different techniques: HPE-PCR, LPCR-SB and SP-PCR. Unlike a conventional PCR, HPE-PCR includes multiple heat pulses during the extension step, which improves the amplification of long CTG fragments. Although it is not commonly used to size the CTG expansion, HPE-PCR is a fast and easy method that showed promising results in a previous publication [81]. On the other hand, LPCR-SB, which consists of a long pre-amplification followed by southern blot hybridization, and SP-PCR, consisting of the use of small amounts of input DNA to amplify few genomic equivalents, are both common techniques currently used to size the expansion in DM1 patients. Moreover, since laboratories are using different primers sets for LPCR, in order to see whether this variability could also affect our results, we not only compared the results between these three techniques, but we also used three different primer pairs for the LPCR-SB assay, resulting in LPCR1-SB, LPCR2-SB and LPCR3-SB. Regarding the CTG expansion, we determined both the estimated progenitor and mode size in each patient for each technique.

HPE-PCR results were inconsistent in our hands since controls showed a similar smear (suggestive of carrying expansions) to the one found in patients. Regarding to LPCR-SB, the amplification of the CTG expansion in some patients was not possible (two for LPCR1-SB and LPCR2-SB, respectively, and 11 for LPCR3-SB). Thus, based on the inconsistency of HPE-PCR and the small amount of data points in LPCR3-SB, the results of both methods were excluded from the statistical analysis. In LPCR-SB, the amplified products in some patients appeared as high smears, probably due to a mobility impairment in long amplifications of highly concentrated DNA (*i.e.*, 100 ng in the final PCR reaction). Consequently, we excluded three data points in LPCR1 that surpassed our detection limit of 2000 CTGs based on the juvenile-classical phenotype of our patients. In the case of SP-PCR, the amplification of the CTG expansion was possible in all 15 patients.

When comparing between the method results, LPCRs yielded shorter progenitor alleles and longer mode alleles compared to SP-PCR. The estimated progenitor allele of LPCR1-SB and LPCR2-SB correlated strongly ($r = 0.983$, $p < 0.0001$), but no correlations were found with SP-PCR. However, the comparison

between the three techniques with analyzable data (*i.e.*, LPCR1-SB, LPCR2-SB and SP-PCR) showed no significant group effect, neither for the CTG expansion size of the progenitor ($p = 0.112$) nor for the mode allele ($p = 0.653$).

Finally, we analyzed possible correlations between the genetic data and the age of disease onset, the Muscular Impairment Rating scale (MIRS) and the modified Rankin Scale (mRS). We found an inverse correlation between the CTG expansion size of the progenitor allele determined by SP-PCR and the age of disease onset ($r = -0.734$, $p = 0.016$).

To better understand the CTG instability present in tissues (**Chapter II**), we analyzed the CTG expansion size in three different tissues (blood, muscle and skin) obtained simultaneously from eight DM1 patients. We determined, by SP-PCR, the estimated progenitor, the most abundant (mode) and the longest CTG expansion size in each tissue, as well as the somatic instability (calculated by subtracting the progenitor CTG size from the mode CTG size). There was one DNA sample from muscle biopsy and three DNAs from skin biopsies which yielded no amplification and precluded the CTG sizing. Thus, we were able to analyze the CTG instability in eight blood, seven muscle, and five skin tissues of our DM1 patients.

Our results indicated that muscle and skin have larger expansions than blood. However, we did not observe significant differences across the tissues in the estimated progenitor ($p = 0.449$), the mode CTG size ($p = 0.247$) and the genetic instability ($p = 0.691$). Instead, we observed that the longest CTG ($p = 0.041$) was significantly different between the tree tissues. Analyzing the CTG distribution in each tissue, we found that in blood, the estimated progenitor, the mode and the longest expansion size were correlated and uniformly distributed (all correlations $p < 0.05$). Contrary, in the case of muscle and skin, only the estimated progenitor and the mode size showed a correlation ($p < 0.05$), whereas the longest size was independent. No correlation was found between tissues. Moreover, as we included a patient carrying variant repeats, we analyzed whether this patient had a more stable CTG distribution in any of the tree tissues. However, we did not observe any difference compared to the CTG distribution of the other patients.

Finally, we aimed to determine whether the genetic data of the different tissues was associated to the patients' phenotype. Thus, we analyzed the relationship of all CTG parameters (estimated progenitor, mode, longest size and genetic instability) with three clinical traits: age of disease onset, Medical Research Council (MRC) scale and MIRS. We found that only the estimated progenitor CTG size in muscle correlated with the age of disease onset ($r = -0.850$, $p < 0.05$). Additionally, the estimated progenitor ($r = -0.932$, $p < 0.05$) and mode size in muscle ($r=-0.898$, $p < 0.05$] correlated with MRC.

We analyzed the presence of variant repeats in 49 Spanish patients belonging to 36 different families (**Chapter III**). We screened the presence of interruptions by TP-PCR technique, which shows the presence of variant repeats as gaps in the contiguous pattern of peaks corresponding to the CTG triplets. In total, five patients of our registry (10%) carried CCG interruptions at the 3'-end of the CTG expansion. These patients belonged to the same family, and were three sisters (P1, P2 and P3) and the son (P4) and the daughter (P5) of two of them.

To determine the interrupted allele patterns of the patients. First, we performed an *Aci*I digestion, an enzyme that specifically cleavages 5' CCGC 3'/ 3' GGCG 5' sequence and thus, allows the detection of CCG or GGC variant repeats. In all five patients, the results showed a downward shift of the smear in the gel of the digested product compared with the nondigested product, indicating that *Aci*I had cleaved the PCR product and therefore the interruption was likely either a CCG or a GGC triplet. Lastly, we sequenced the DNAs using specific CCG and GGC primers to reveal the detailed interrupted pattern. Two patients, mother (P2) and son (P4), showed the same interruption pattern, consisting in a complex CCG pattern with one pair of CCGs together with other isolated CCG repeats. The other three family members had different interruption patterns. Patient P1 had isolated CCG repeats scattered across the expansion. Patient P3 showed a few CCGCTG hexamers inside a complex pattern with some extra CCG located in different positions. Patient P5 (the daughter of P3) showed a pattern similar to that of her mother with respect to the hexamers, but with some extra CCGs located in different positions, generating three consecutive CCG repeats.

We measured by SP-PCR the estimated progenitor allele size of the five patients carrying variant repeats. Comparing their estimated progenitor allele size, we could determine the effect of the interruptions on the expansion inheritance between generations. We detected a contraction in the repeat size from patient P2 to patient P4 (*i.e.*, from mother to son) and an expansion from patient P3 to patient P5 (*i.e.*, from mother to daughter). This expansion was also linked to anticipation, with an earlier age of onset for P5 compared to her mother (P3).

Regarding the phenotype, the three sisters had a late onset of symptoms (≥50 years). Patient P1 was the oldest of the siblings and the most severely affected, presenting atypical traits such as severe axial and proximal weakness requiring walking assistance. Patients P1 and P2 showed severe cardiac and respiratory dysfunction. Patient P3 showed cardiac dysfunction and had severe axial weakness as well, with mild proximal limb weakness and moderate distal weakness. Patient P4, at the age of 35 years, was asymptomatic and had no detectable myotonia or cardiac alterations. Contrary, patient P5, started with clinical manifestation at the age of 27. Five years later, P5 was displaying classic DM1 traits, such as facial weakness and cardiac impairment.

We aimed to analyze the main molecular players that are involved in the toxic RNA gain-of-function pathomechanism (**Chapter IV**). To do that, we used a 3D imaging technology by which we analyzed 71 whole myoblasts derived from DM1 patients. First, we divided the cultured patients' myoblasts in two different pools. One of these pools was used to determine, by LPCR-SB, the CTG expansion size present in the patients' myoblasts, which ranged from 195 to 1568 CTG repeats. The other cell pool was used to stain RNA foci and MBNL1 protein and subsequently perform the 3D imaging analysis. The 3D imaging was obtained through several Z-stack images covering the total thickness of the myoblasts and further processing with Zen Black and Imaris.

The number of RNA foci was highly heterogeneous among the myoblasts, not only between the myoblasts from different patients, but also between the myoblasts coming from the same patient. The average number of RNA foci found in our 71 myoblasts was of 3 RNA foci per cell, in a range from 0 to 6. Of note, two patients only

displayed RNA foci in 40% of the myoblasts, whereas the other four patients had RNA foci in 100% of their myoblasts. When we compared the size of the CTG expansion with the number of RNA foci present in the myoblasts, we found a strong positive correlation ($r = 0.876$, $p = 0.022$).

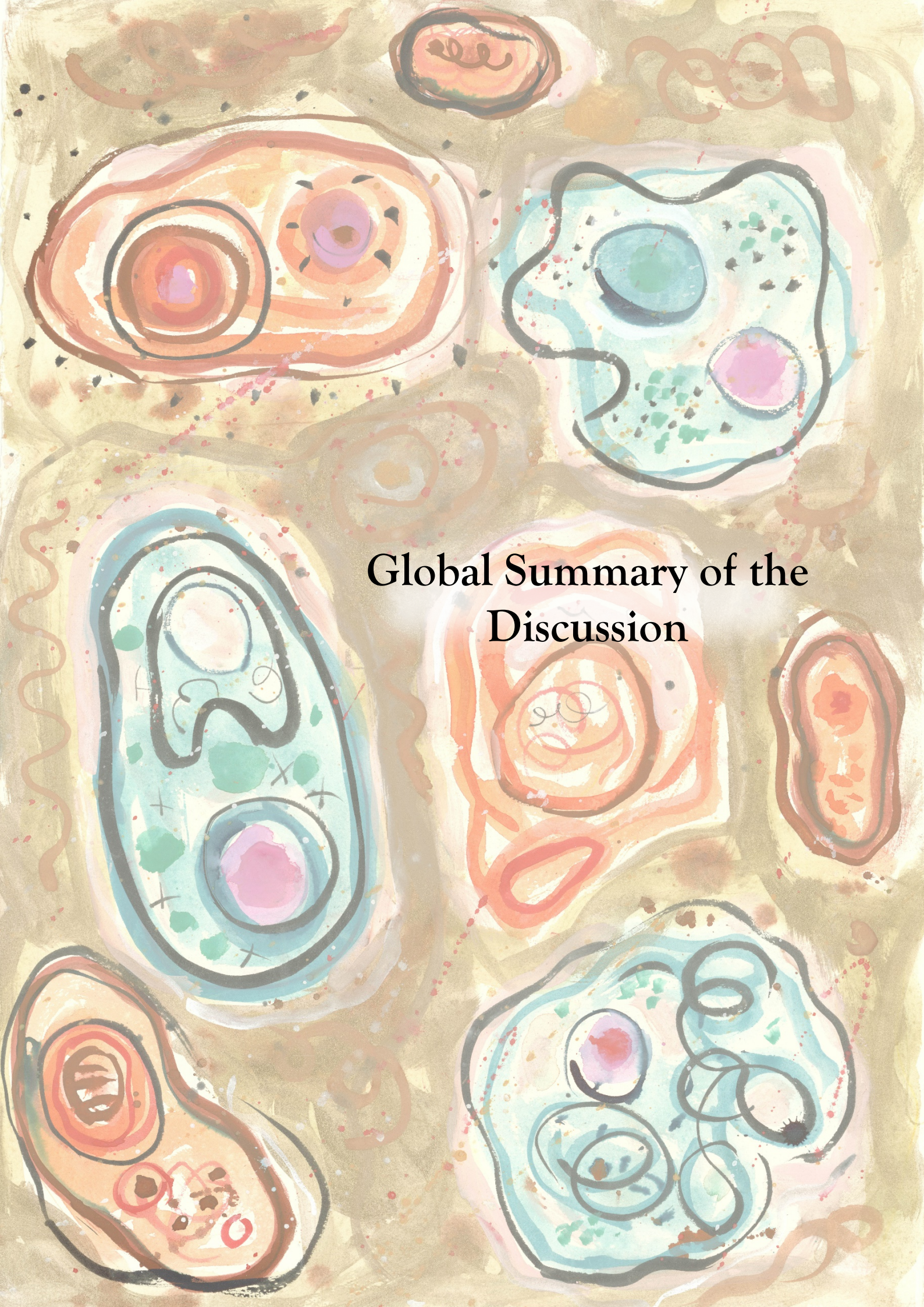
Regarding the localization of the RNA foci, we found that most of these RNA foci were located in the nucleus of the cells. However, 12% of the total RNA foci found in the 71 myoblasts were located in the cytoplasm varying in a range from 4% to 23% in the different DM1 patient lines. All 6 DM1 myoblast cell lines showed presence of RNA foci in the cytoplasm in some cells. The percentage of cells carrying cytoplasmic RNA foci varied among the DM1 patients' lines in a range from 10% to 45% of the cells. Actually, we found a strong, positive correlation between the number of CTG repeats and the percentage of cells with cytoplasmic RNA foci ($r = 0.943$, $p = 0.017$). Interestingly, the percentage of cells with cytoplasmic RNA foci was also correlated to the percentage of cells carrying RNA foci (nuclear and/or cytoplasmic) ($r = 0.845$, $p = 0.033$), which also correlated with the average number of RNA foci (nuclear and/or cytoplasmic) per myoblast ($r = 0.886$, $p = 0.033$). Moreover, we analyzed the area occupied by RNA foci and found that those cell lines that contained RNA foci with larger areas had more foci in the cytoplasm ($r = 0.839$, $p = 0.037$). When comparing the molecular data that we obtained with the clinical characteristics of the patients, we found that a higher number of cytoplasmic RNA foci is associated with an earlier disease onset ($r = -0.818$, $p = 0.047$).

We further calculated the volume that the nuclear RNA foci occupied compared to the total volume of the nucleus, and we found that RNA foci only represented a small fraction (0.34 to 0.53%) of the total nucleus volume.

Regarding the MBNL1 protein, we observed that it was located both in the nucleus and in the cytoplasm of DM1 myoblasts. The colocalization with RNA foci was present in all patients' cell lines and not only with the nuclear RNA foci but also with the cytoplasmic RNA foci. Actually, the cytoplasmic colocalization represented 9% of the total cellular colocalization. When we analyzed the amount of trapped MBNL1, we found that it represented less than 1% of the total MBNL1 expression in DM1 myoblasts.

As RNA foci are the product of *DMPK* gene transcription, we aimed to analyze the expression of DMPK transcripts. Therefore, we performed quantitative-PCR using a probe that was located outside the CTG expansion, thus, detecting the expression of both wild-type and expanded *DMPK* alleles. We did not find significant differences between the *DMPK* gene expression of patients and controls myoblasts. However, we found a positive correlation between *DMPK* gene expression and both the nuclear RNA foci ($r = 0.993$, $p = 0.008$) and the total (nuclear and/or cytoplasmic) RNA foci number ($r = 0.967$, $p = 0.033$).

Finally, to study how the alternative splicing was affected in DM1 myoblasts, we analyzed the expression of the inclusion and exclusion isoforms—INSR exon 11, ATP2A1 exon 22, and MBNL1 exon 7—. However, we did not detect the typical DM1 splicing misregulation in myoblast.



Global Summary of the Discussion

Global Summary of the Discussion

Regarding the **Chapter I**, although no significant group effect was found between the techniques that are used to size the CTG expansion, there is variability in the number of CTG repeats for a given patient depending on the CTG sizing method. Regarding the HPE-PCR, we obtained results that were inconsistent and thus, difficult to interpret. The fact that LPCR1-SB and LPCR2-SB yielded correlated results only regarding the estimation of the progenitor allele size, means that when amplifying short CTG expansions, the variability between these two methods is lower than when amplifying longer repeats. Thus, the LPCR-SB technique would be sensitive to the size of the template. Additionally, LPCR-SB might be more sensitive to parameters, such as the quality of input DNA, compared to SP-PCR, since LPCR3-SB did not allow the amplification of most of the patients´ DNA samples.

Using high input of DNA in LPCR-SB approaches, together with the instability of the mutation, hinders the differentiation of the progenitor allele size from possible contractions of the repeat. Another factor that may affect the results is the number of PCR cycles—35 cycles are used in LPCR vs. 28 for SP-PCR—since a high number of cycles facilitates the amplification of shorter products, whereas longer products may not be favored. Moreover, the number of PCR cycles increases PCR slippage, which tends to shorten the products. These phenomena could explain why LPCR-SB yielded lower progenitor allele sizes than SP-PCR. By contrast, SP-PCR—which amplifies only small input DNA—shows discrete bands that allow for a detailed analysis of the mutational spectrum and allele size distribution [86]. As such, this technique enables a better detection and estimation of the progenitor allele from post-contractions of the repeat.

SP-PCR was the only technique that enabled amplification of all DNA samples and in fact, was the only one yielding a result (the progenitor allele size) that was correlated with an important phenotype trait of DM1, the age of disease onset. Thus, our results suggest that progenitor allele length is the major modifier of age of disease onset, and as such, it is very important to use an accurate method for its determination. Furthermore, the results are overall in accordance with previous studies reporting a correlation between progenitor allele length measured by SP-PCR and both age of disease onset and clinical severity [70,80], although we found no correlation with MIRS

or mRS. The size of the cohort plus the fact that CTG sizes in blood may be poor representatives of muscle status, might explain the lack of finding these correlations.

There are many studies that are published nowadays that do not detail the technical approach that has been followed to size the CTG expansion, neither the criteria that they used to select the band from which the CTG expansion size is calculated. In this sense, our study shows how important it is to share this information, in order to be able to compare studies and to understand the process that has been followed. Moreover, it is a priority to generate a consensus among researchers working in DM1, to decide which is the referral technique to use for sizing the CTG expansion in patients with DM1.

Analyzing the CTG expansion in three different tissues (**Chapter II**), our results showed that CTG expansions are in general larger in muscle and skin than in blood. These results are in accordance to previous studies in which they showed that muscle [61,62,66] and patients' fibroblasts [63] carry larger expansions than lymphocytes. However, no previous study has assessed simultaneously the CTG repeats in blood, muscle and skin tissues by SP-PCR, considering the CTG instability present in the tissue and not only measuring one single CTG size. This strategy allowed us to determine that between tissues, the progenitor and the mode size do not differ significantly, but the longest expansion does. This finding suggests that the CTG track is expanding in different manners in each tissue during development, and that the variability of the CTG expansion increases with time.

We also observed that the genetic instability was present in the three tissues. Genetic instability is a complex phenomenon that can be produced by many factors [74,256–259], not only during cell division, but also during DNA repair events, transcriptions and epigenetic changes. In muscle and skin, a non-dividing cell status and the DNA repair mechanisms probably play an important role in its instability. In the case of blood, the instability is most likely also affected by the dividing status of these cells.

Analyzing each tissue independently, we found that all measures in blood (progenitor, mode and longest CTG) are correlated, suggesting that in blood, the progenitor CTG size leads the expansion mosaicism. Conversely, in muscle and skin

the progenitor correlated with the mode but not with the longest CTG, meaning that the expansion mosaicism of the CTG track in these tissues is more random, probably because of the longer repeat lengths.

We did not find differences between the CTG instability in the patients carrying variant repeats compared to the rest of the patients who carried a pure CTG expansion. Some authors have described that the variant repeats might have a stabilizing effect on the CTG expansion [75,79]. We analyzed for the first time the genetic instability in different tissues of a patient carrying variant repeats, and we did not find a more stable CTG distribution compared to the other patients carrying a pure CTG expansion.

We found that the muscle progenitor allele was the only one associated with age of disease onset. It has been shown that the progenitor size present in blood of DM1 patients is also a good indicator of age of disease onset [70]. However, probably due to the small sample size of the study, we could not find this correlation in this cohort. The fact that we did find correlations with the CTG expansion size present in muscle is not surprising considering that it is one of the most affected tissues in DM1. Our results have confirmed the hypothesis of different authors suggesting that CTG measured in muscle could correlate better with disease severity than CTG measured in blood [61,62]. Our data not only indicates that the CTG size present in muscle correlates better with the age of disease onset than the present in blood, but also that it correlates with the MRC scale in patients' muscles. These results indicate the direct involvement of the CTG expansion in muscle function, compromising patients' muscle strength.

The effect of variant repeat patterns on the DM1 clinical phenotype is still unclear (**Chapter III**). Previous studies have led to the believe that patients with interrupted alleles have some atypical symptoms, but overall a milder phenotype than their age matched DM1 non-interrupted peers with a similar repeat length [42,72,75,77,259]. In this respect, our results support the occurrence of atypical DM1 features and late age of onset, but not of a milder phenotype in patients carrying interruptions. Indeed, several classical symptoms could be found in some members of our family, and P4 was still asymptomatic at age 35. The majority of the patients

however, showed a more severe manifestation of the disease, including cardiorespiratory problems and also needing mechanical ventilation and a pacemaker. In addition to the severity of the disease, they also presented an atypical distribution pattern of muscle weakness; axial weakness and mild or absent facial weakness that could confuse the DM1 diagnosis for the diagnostic of other dystrophies instead. We should take into account that contrary to the vast majority of the studies, we analyzed patients carrying interruptions after several years since the beginning of their symptoms. This fact could explain why we did find patients carrying variant repeats with a significant disease severity compared to other authors, who reported milder symptoms. Thus, despite the small number of patients within the same family, our results challenge the notion that interrupted patients who remain asymptomatic until their late 30s or 40s are not at risk for having a severe phenotype later in life.

The prevalence of interrupted alleles among our patients was ~10%, and 3% among the studied DM1 families. This is in overall agreement with previous studies in which the prevalence in families ranged from 3% to 5%. The type of interruption present in our cohort was CCG, which is currently the most frequently reported variant repeat. We also found that the CCG repeat pattern can vary or be maintained between intergenerational transmissions.

In between generations, we detected a contraction of the expansion between mother (P2) and son (P4), but also an expansion between mother (P3) and daughter (P5). Previous studies suggest that CTG expansion containing variant repeat patterns display more frequently stable, or even contracted DMPK alleles, instead of further expanded DMPK alleles [42,72,74,75,77]. However, they also found anticipation in interrupted DM1 families [72,75,77]. Perhaps these expansions in interrupted alleles from one generation to another are less frequent than in pure CTG expansions transmissions, but they do occur.

The small number of interrupted patients present in the DM1 population makes it hard to perform genotype-phenotype correlations and there is still much uncertainty. Studies with larger DM1 cohorts, preferably with DM1 families, are needed to unravel the phenotypic consequences of variant repeat patterns and to study their effect on intergenerational transmissions of the DMPK expanded allele.

We analyzed molecular factors that are involved in the main pathological mechanism underlying DM1 (**Chapter IV**). First, our results showed that the number of RNA foci per DM1 myoblast is regulated by the CTG expansion length. A previous study also showed that the number of RNA foci is linked to the CTG expansion size. They divided DM1 patients in two groups, patients who carried less than 500 CTGs and patients who carried more than 1000 CTGs, and they found that the group with the higher CTG expansion size had a higher RNA foci number compared to the group carrying lower CTGs [123]. We found that this relationship is maintained in a broader range of CTG expansions, not dividing the patients in groups but studying the correlation in a cohort of patients who carried from 192 to 1568 CTG repeats.

Our data indicated that there is a small number of RNA foci per DM1 myoblast, averaging only 3 per cell (range 0 to 6), which is consistent with the findings of previous research in muscle tissue [123,124,260]. Interestingly, not all the DM1 myoblasts derived from the same patient had RNA foci. Two lines of patient-derived myoblasts, who carried the shorter expansions, showed RNA foci only in 40% and 80% of the cells. Thus, our results suggest that CTG expansion not only regulates the amount of RNA foci per myoblast, but also the number of myoblasts presenting RNA foci.

We found both nuclear and cytoplasmic RNA foci. CTG expansion size appears to regulate the presence of cytoplasmic RNA foci. Thus, larger CTG expansions will produce a higher number of nuclear foci and therefore more cells will contain cytoplasmic foci because the latter only appeared in association with nuclear foci. The presence and origin of cytoplasmic foci have not yet been studied in-depth. Some authors have suggested that cytoplasmic RNA foci are a product of the cell cycle as well as of the breakdown of the nuclear membrane, and as such are more likely to appear in dividing cells [132]. However, one study in a DM1 mouse model expressing CTG expansions in the heart tissue found RNA foci exclusively in the cytoplasm of cardiomyocytes, which are highly-differentiated, non-dividing cells [261]. Furthermore, our results showed that cytoplasmic RNA foci colocalize with MBNL1. The role of cytoplasmic foci, and whether they have a toxic effect should be studied in future research.

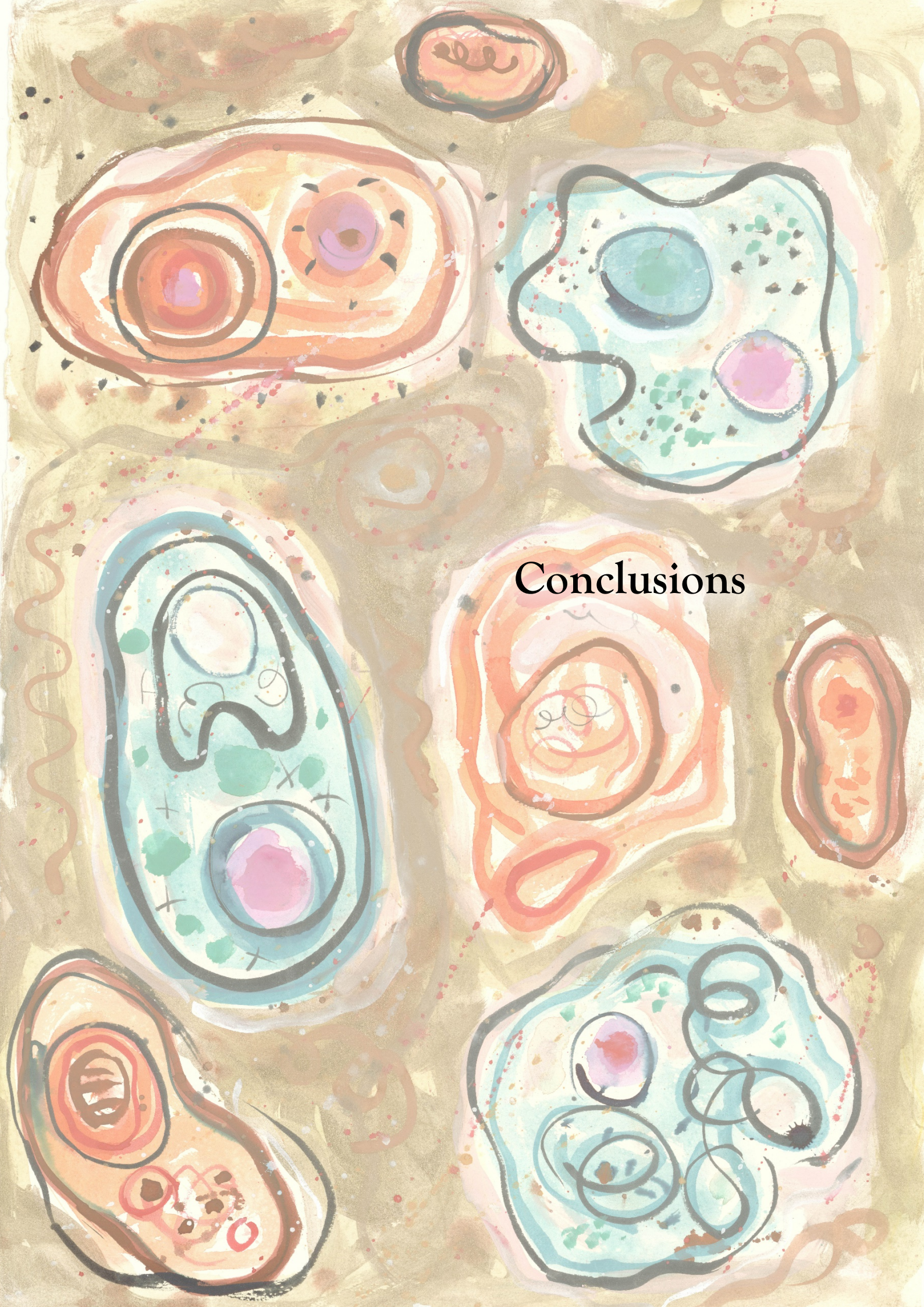
RNA foci represent less than 0.6 % of the total nuclear volume in DM1 myoblasts. These data suggest that the impact that RNA foci have on the nuclear volume is minimum. Additionally, when analyzing the area of RNA foci in relation to other molecular findings, we observed that bigger areas are related to a higher number of cytoplasmic foci. The fact that scarce data are currently available on cytoplasmic RNA foci makes it difficult to interpret our results. However, as previously described by other authors, RNA foci can fuse or divide randomly with no apparent or known purpose [130].

Our analysis of MBNL1 revealed that the sequestration of this protein inside RNA foci represents less than 1% of the total MBNL1 myoblast expression. Although there are studies showing the pathological role of MBNL1 in DM1 [137,138,262–264], our results are consistent with a previous study by Coleman *et al*. reporting that only a small proportion of MBNL1 protein (0.2%) was sequestered by RNA foci in DM1 lens epithelial cells [265]. In this context, the low values of sequestration of MBNL1 would suggest that this phenomenon plays only a minor role in the pathophysiology of the disease in these cells, as 99% of total MBNL1 expression is free.

The levels of *DMPK* expression were related to the number of RNA foci per cell. Indeed, if there are more RNA foci inside a cell, theoretically this should be related to a higher level of *DMPK* expression. Moreover, we used quantitative-PCR for determining the *DMPK* expression, which allows detecting the expanded as well as the wild type allele.

We did not find the expected altered DM1 splicing when analyzing IR, ATP2A1 and MBNL1 transcripts. These results could be related to the fact that the expression of the aforementioned aberrant isoforms is related to developmental stages, and since we used undifferentiated cells (*i.e*., myoblasts), the expression pattern could differ from the pattern of fully differentiated myotubes, and especially of skeletal muscle fibers.

3D imaging allowed us to study the total number of RNA foci present in myoblasts derived from DM1 patients, their area and localization, as well as the colocalization with MBNL1. We have been able to analyze different DM1 molecular players, identifying correlations between them and contributing to the understanding of the pathomechanism in DM1.



Conclusions

Chapter I

LPCR-SB and SP-PCR generate different results in terms of sizing the CTG expansion in DM1 patients. This is probably affecting the possible establishment of genotype-phenotype correlations.

SP-PCR seems to be the most suitable method to size the CTG expansion in DM1 patients. It is less sensitive to the DNA quality, it provides specific data of the allele distribution (including progenitor, mode and longest allele), and the progenitor allele determination is related with the age of disease onset.

An international agreement among the DM1 community is needed to determine which is the most suitable methodology to characterize the CTG expansion size in patients with DM1.

Chapter II

CTG instability is present in blood, muscle and skin tissues of patients with DM1, with larger expansions present in muscle and skin than in blood.

The estimated progenitor CTG size in muscle is a good predictor of disease age of onset and muscle strength.

Chapter III

DM1 patients carrying interruptions may have classical symptoms as the ones found in DM1 patients with pure repeats, including cataracts, myotonia or muscle weakness. However, patients carrying interruptions may also present other atypical symptoms which can hamper the diagnosis, such as mild or absence of facial weakness, and severe axial weakness.

The presence of interruptions can be associated to a late age of onset although the phenotype may not be mild. Therefore, even though the disease can appear later in life, these patients require clinical follow-up and genetic counseling similar to the patients carrying pure repeats.

The presence of interruptions can lead to a contraction of the CTG repeats but also to an expansion linked to anticipation.

Chapter IV

3D molecular imaging appears to be a potential tool to identify possible associations between molecular alterations and clinical manifestation in DM1 patients.

The CTG expansion determines the number of RNA foci per myoblast and the number of myoblasts that contain RNA foci, as well as the appearance of cytoplasmic foci.

RNA foci only represent a negligible part of the total nucleus volume.

The fraction of trapped MBNL1 in RNA foci is minor compared to the total amount of MBNL1 present in myoblasts derived from DM1 patients.

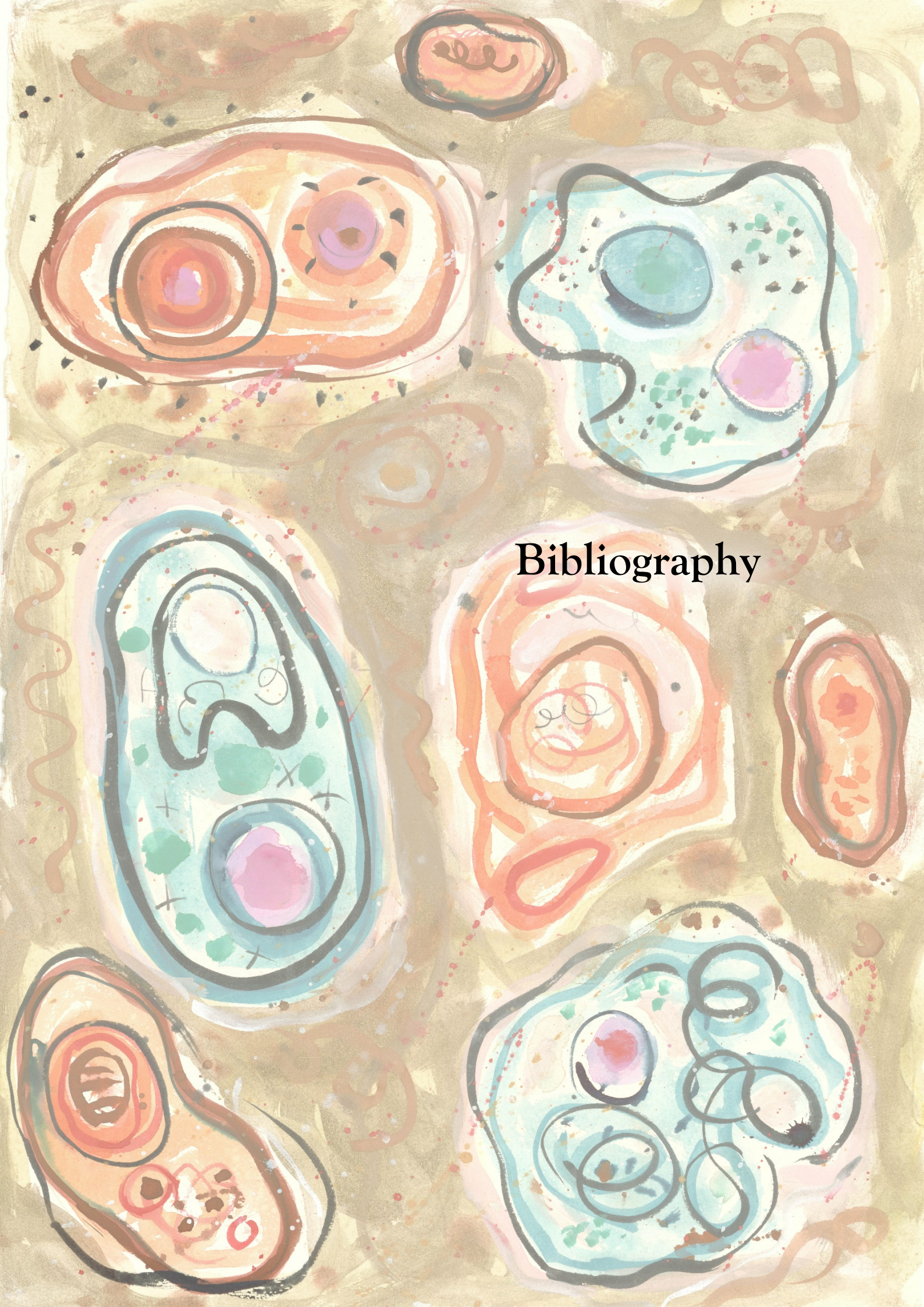
Cytoplasmic RNA foci should be considered in future studies in order to clearly elucidate their role in the pathobiology of DM1.

Chapter V

BNA^{NC} treatment has different efficacy (RNA foci reduction) and toxicity (cell mortality) in fibroblasts, lymphoblasts and myoblasts derived from DM1 patients.

Myoblasts, derived from one of the most affected tissues in DM1 patients, have a lower response to the treatment compared to fibroblasts and lymphoblasts. New experiments improving the delivery in these cells should be performed.

The efficacy of the treatment in terms of the RNA foci reduction seems to not be related to the CTG expansion size.



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