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Departamento de Ciências Médicas

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Caracterização das alterações do envelope nuclear na Distrofia Miotónica Tipo 1

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biomedicina Molecular realizada sob a orientação científica da Professora Doutora Sandra Maria Tavares da Costa Rebelo, Professora Auxiliar Convidada do Departamento de Ciências Médicas da Universidade de Aveiro e coorientação da Doutora Filipa de Sá Martins, Investigadora de pós-doutoramento do Departamento de Ciências Médicas da Universidade de Aveiro.

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palavras-chave

Distrofia miotónica tipo 1, proteínas do envelope nuclear, funções nucleares, fibroblastos humanos

resumo

A distrofia miotónica tipo 1 (DM1) é uma disfunção genética multissistémica associada principalmente à degeneração do músculo esquelético e causada por uma expansão repetida de trinucleótidos CTG na região 3' não traduzida do gene *DMPK*, acumulando-se na forma de inclusões nucleares e comprometendo a função nuclear. Nos últimos anos, alguns estudos associaram algumas proteínas do envelope nuclear (NE) como sendo componentes importantes na resposta das células ao stress mecânico, ajudando a manter a integridade das células musculares, o que demonstra uma associação com a DM1. De facto, já foi demonstrado que os fibroblastos de pacientes com DM1 apresentavam uma estrutura nuclear comprometida e uma localização alterada de algumas proteínas do NE. No entanto, a contribuição que o envelope nuclear possa ter para a DM1 ainda não foi totalmente elucidada. Neste trabalho, realizámos um estudo espectroscópico ATR-FTIR usando fibroblastos humanos de controlos e de pacientes com DM1 de forma a identificar diferenças espectrais. Os fibroblastos dos pacientes incluídos neste estudo têm 1000 repetições de CTG e 2000 repetições de CTG, representando os tipos DM1 adulto e congénito, respectivamente desta doença. Os modelos de DM1 usados neste estudo apresentaram diferenças a nível molecular, principalmente nas estruturas das proteínas e dos lipídos: os modelos de DM1 podem ser diferenciados dos controlos através de cadeias lipídicas maiores, cadeias lipídicas mais saturadas e presença de agregados proteicos. Estas diferenças podem ser explicadas por um metabolismo lipídico alterado e pela acumulação de agregados tóxicos sob a forma de RNA e de proteínas de poliglutaminas expandidas. Além disso, foi avaliado o perfil nuclear em fibroblastos humanos de controlos e de pacientes com DM1, de forma a caracterizar algumas características nucleares específicas, nomeadamente: área nuclear, circularidade, deformações (*blebs* e núcleos irregulares) e presença de micronúcleos. Os nossos resultados evidenciaram uma tendência para a área nuclear, o número de células com micronúcleos e os núcleos deformados aumentarem nos modelos de DM1, em comparação aos controlos. Além disso, para observar a localização de algumas proteínas do envelope nuclear, nomeadamente, lamina A/C, emerina, SUN1 e LAP1, foi realizada uma análise imunocitoquímica nos fibroblastos humanos de controlos e de pacientes com DM1. Em relação à lamina A/C, emerina e LAP1, estas proteínas apresentaram uma localização alterada no envelope nuclear em ambos os modelos de DM1 (DM1 1000 e DM1 2000). Além disso, a lamina A/C e a emerina mostraram estar acumuladas nas inclusões nucleares marcadas por estas proteínas, enquanto que a SUN1 e a LAP1 mostraram estar acumuladas nas invaginações nucleares marcadas por estas proteínas. Para concluir, foram identificadas diferenças espectrais entre os controlos e os modelos de DM1. Além disso, a arquitetura nuclear e as proteínas do envelope nuclear mostraram estar alteradas nos modelos de DM1. Portanto, mais estudos devem ser realizados para elucidar a contribuição que as proteínas do envelope nuclear possam ter para esta doença.

keywords

Myotonic dystrophy type 1, nuclear envelope proteins, nuclear functions, human fibroblasts

abstract

The myotonic dystrophy type 1 (DM1) is a multisystem genetic disorder mainly associated with degeneration of skeletal muscle and caused by a CTG repeat expansion in the 3′ untranslated region of the *DMPK* gene, accumulating as nuclear foci and compromising the nuclear function. In the last few years, some studies associate nuclear envelope proteins as a significant component in the response of cells to mechanical stress, helping to maintain muscle cell integrity, demonstrating a clear association to DM1 pathology. In fact, it has been demonstrated that DM1 patient's fibroblasts presents an impaired nuclear structure and an altered localization of some NE proteins. However, the contribution of the NE for DM1 was not fully elucidated. In this work, we performed an ATR-FTIR spectroscopy study of human control and DM1 fibroblasts to identify spectral differences. The human patients' fibroblasts included in this study have 1000 CTG and 2000 CTG repeats, representing the adult and congenital DM1 types, respectively. DM1 fibroblasts present differences at the molecular level, mainly in protein and lipids structures: DM1 donors can be distinguished from controls by a larger lipidic stretching, more saturated lipidic stretching and presence of protein aggregates. These differences can be explained by the dysregulation of lipins in DM1, affecting the lipid metabolism, and RNA and polyglutamine expansion proteins accumulation as nuclear aggregates, respectively. Moreover, the nuclear profile in human fibroblasts derived from DM1 patients was evaluated, in order to characterize some particular nuclear features, namely the nuclear area, circularity, deformations (blebs and misshaped nuclei) and micronucleus presence. Our results evidenced a tendency to increase the nuclear area, the number of cells with micronuclei and the % of deformed nuclei in DM1 donors, comparing to the control. Further, in order to unravel some nuclear envelope proteins alterations in DM1, namely in lamin A/C, emerin, SUN1 and LAP1, we performed immunocytochemistry analysis in human fibroblasts. Regarding lamin A/C, emerin and LAP1, these proteins showed an altered localization in nuclear envelope, in both DM1 fibroblasts. Also, both lamin A/C and emerin are highly present in protein positive-nuclear inclusions whereas SUN1 and LAP1 are highly present in protein positive-nuclear invaginations. To conclude, spectral differences have been identified between controls and DM1 fibroblasts. Moreover, the nuclear architecture and nuclear envelope proteins seems to be altered in DM1 donors. Therefore, further studies should be performed in order to elucidate nuclear envelope proteins contribution to the disease.

List of abbreviations

A.U. – Arbitrary Units

A-EDMD - Autosomal dominant form of Emery-Dreifuss muscular dystrophy

ANOVA – Analysis of variance

ATR - Attenuated total reflectance

ClC-1 - Muscle chloride channel

CNBP/ZNF9 - CCHC-type zinc finger nucleic acid binding protein

CNS - Central nervous system

cTNT - Cardiac troponin T

CUG-BP - CUG RNA binding protein

DAG - Diacylglycerol

DAPI – 4',6-diamidino-2-phenolyde

DCM-CD - Dilated cardiomyopathy with conduction-system disease

DM - Myotonic dystrophy

DM1 – Myotonic dystrophy type 1

DM2 - Myotonic dystrophy type 2

DMD - Duchenne Muscular Dystrophy

DMEM – Dulbecco's Modified Eagle Medium

DMPK - Myotonic dystrophy protein kinase

DNA – Deoxyribonucleic acid

DYT1 – Dystonia early-onset torsion dystonia

ECM – Extracelular matrix

EDMD – Emery-Dreifuss muscular dystrophy

ER – Endoplasmic reticulum

FBS – Fetal Bovine Serum

FG - Phenylalanine-glycine

FTIR - Fourier Transform Infrared Spectroscopy

HGPS - Hutchinson–Gilford progeria syndrome

HRP – Horseradish peroxidase

ICC – Immunocytochemistry

Ig – Immunoglobulin

INM – Inner nuclear membrane

IR - Insulin receptor

LAP1 – Lamina-associated polypeptide 1

LAP2 – Lamina-associated polypeptide

LBR – Lamin B receptor

LGMD1B - Limb–Girdle muscular dystrophy type 1B

LiM – Light microscopy facility

LINC – Linker of nucleoskeleton and cytoskeleton

MAN1 – Inner nuclear membrane protein Man1

MBNL - Muscleblind like splicing regulator protein

min – minutes

MIR - Mid-infrared

NE - Nuclear envelope

NES - Nuclear export signal

NLS - Nuclear localization signal

NPC – Nuclear pore complex

NUP – Nucleoporin

ONM – Outer nuclear membrane

PA - Phosphatidic acid

PBS – Phosphate buffered saline

PC - Phosphatidylcholine

PDM - Proximal myotonic dystrophy

PE – Phosphatidylethanolamine

PKC - Protein kinase C

PN – Passage number

PNS – Perinuclear space

polyQn - Polyglutamine expansion proteins

PP1 - Protein phosphatase 1

PROMM - Proximal myotonic myopathy

PTMs - Post-translational modifications

Rb - Retinoblastoma

RNA – Ribonucleic acid

rpm – rotations per minute

RT – Room temperature

SEM – Standard error

SUN1 – Sad1/unc-84 protein-like 1

TAG - Triacylglycerol

TGFβ – Transforming growth factor beta

TNNT2 - Troponin T2

TNNT3 - Troponin T3

TOR1AIP1 – Torsin-1A-interacting protein 1

UTR - Untranslated region

X-EDMD - X-linked form of Emery-Dreifuss muscular dystrophy

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CHAPTER I: Introduction

1.1. The Myotonic Dystrophy

The muscular dystrophies are inherited disorders caused by mutations in different genes. These disorders include myotonic dystrophies, congenital, Duchenne, Becker, Emery-Dreifuss, facioscapulohumeral, oculopharyngeal and limb-girdle muscular dystrophies [1]. Their common features are progressive weakness and degeneration of skeletal muscle, however, each disorder may differ in age of onset, disease severity, pattern of inheritance and affected tissues [2]. The myotonic dystrophy (DM) is a progressive autosomal dominant disease which can be divided into two genetically distinct forms [3]: the myotonic dystrophy type 1 (DM1), also known as Steinert's disease [3], and the myotonic dystrophy type 2 (DM2), also known as proximal myotonic myopathy (PROMM) [4] or proximal myotonic dystrophy (PDM) [5]. DM1 and DM2 are caused by similar mutations, a $(CTG)_n$ expansion in the myotonic dystrophy protein kinase (*DMPK)* gene and a $(CCTG)_n$ expansion in the CCHC-type zinc finger nucleic acid binding protein (*ZNF9/CNBP*) gene, respectively [6–8]. These mutations have been identified predominantly in European Caucasians and, in the United States, DM2 is less common than DM1 [9,10]. DM1 is the most common form of muscular dystrophy in adults, with an estimated global prevalence of 1 in 8000 individuals [11,12].

1.1.1. Clinical features

The myotonic dystrophies are considered progressive and multisystemic disorders. However, the presence of myotonia is recognized as a distinctive aspect and a hallmark of these diseases and there is currently no cure [3,13,14]. Therefore, DM1 and DM2 are associated not only with skeletal muscle impairments, predominantly weakness, myotonia (sustained muscle contraction leading to stiffness) and atrophy (muscle wasting), but also with multisystem features, including cognitive impairment, cardiac arrhythmia and cataracts [3,13–15].

DM1 and DM2 share many phenotypic features, although also have key characteristics that distinguish the two type of patients (presented in Table I.1). DM1 is characterized by a more distal pattern of muscle weakness [14], the manifestation of cataracts before age 50 years [16] and the main causes of death are cardiorespiratory disorders, which are responsible for 70% of the mortality in this type of disease [17,18]. On the other hand, DM2, which is a clinically milder disease than DM1 [15], is characterized by a more proximal pattern of muscle weakness [14] and overall less severe cardiac, respiratory, and central nervous system (CNS) involvement [19]. Also, the most common symptom reported in DM2 is muscle pain (myalgic pain) [19], including

abdominal, musculoskeletal, and exercise-related pain, which compromises the quality of patient's life [15,20].

Table I.1 – Main clinical differences between DM1 and DM2. Adapted from Meola *et al.* [13]

Clinical features		**DM1**	**DM2**
General features	Epidemiology	Widespread	European
	Life expectancy	Reduced	Normal
Core features	Myotonia	Evident in adult-onset	Present in <50%
	Muscle weakness	Disabling at age 50	Onset after age 50–70
Muscle symptoms	Distal limb muscle weakness	Always prominent	Absent or mild
	Proximal limb muscle weakness	Absent or mild	Always prominent
	Myalgic pain	Absent	Present in ≥50%
	Facial and jaw weakness	Present	Usually absent
	Bulbar weakness-dysphagia	Present	Usually absent
	Respiratory muscles weakness	Present	Exceptional
	Sternocleidomastoid weakness	Face, temporal, distal hands and legs	Usually absent
Systemic features	Tremors	Absent	Prominent
	Hypersomnia	Prominent	Infrequent
	Cognitive disorders	Prominent	Absent
	Cardiac arrhythmias	Always prominent	From absent to severe
	Male hypogonadism	Manifest	Subclinical in most
	Manifest diabetes	Frequent	Infrequent
	Cataracts	Always prominent	Present in minority

DM1 shows a diverse set of symptoms and wide variability of disease onset, often resulting in misdiagnosis or delay in diagnosis [3,21]. In this way, to provide a framework for recognition and prognosis, there are different phenotypic groups identified in DM1 patients: pre-mutation, congenital-onset, childhood-onset, adult-onset, and late-onset (presented in Table I.2) [13,22,23]. The best characterized group is adult-onset, which is associated with muscle weakness, myotonia and atrophy, cataracts, CNS, gastrointestinal and endocrine dysfunction, cardiac conduction abnormalities, sleep disorders and shortened lifespan [14,24]. Congenital-onset is associated with severe hypotonia at birth, significant intellectual impairment, feeding difficulties and a high frequency of respiratory insufficiency, leading to death in about 30% in the neonatal period [14,25,26]. Both childhood-onset and juvenile-onset are characterized by cognitive dysfunction, motor and cardiac abnormalities. However, the juvenile-onset typically has fewer motor and cardiac symptoms [14,26]. Late-onset is associated with cataracts, less severe muscle involvement and a normal lifespan [14]. Patients with premutations are asymptomatic but are a high risk of transmitting an expanded mutant allele to offspring. There is a clear difference between DM1 and DM2 phenotypic groups since onset of DM2 only occurs in adult ages with no reports of congenital onset [3,14].

Table I.2 – Phenotypic groups of myotonic dystrophy type 1. Adapted from LoRusso *et al.* [14]

Phenotypic group	Age of onset	CTG repeats
Congenital-onset	Apparent at birth	> 1000
Childhood-onset	< 10 years	50 – 1000
Juvenile-onset	10 – 20 years	50 – 1000
Adult-onset	20 – 40 years	50 – 1000
Late-onset	> 40 years	50 – 100
Pre-mutation	Not applicable	38 – 49

1.1.2. DM1 genetics

DM1 is an inherited autosomal dominant disease that is passed to offspring's through a faulty gene [3,13,14]. The gene mutation involves an expansion of a CTG repeat sequence that is transcribed into RNA but not translated, in the 3'UTR (untranslated region) of the myotonic dystrophy protein kinase (*DMPK*) gene, located at the chromosome 19q13.3 [8,13,27]. There is a direct correlation between CTG repeat expansions, the age of onset and severity of disease, since larger repeat expansions present an earlier onset and more severe symptoms [13,28]. The normal length for the described trinucleotide expansion is between 5-37 CTG repeats, while the pre-mutation is associated with 38-49 CTG repeats. Affected patients can have between 50-1000 CTG repeats and the congenital-onset is considered the most severe form of the disease, with expansions of > 1000 CTG repeats [22,29] (Figure I.1). The presence of genetic anticipation and somatic mosaicism occurs in DM1 because of the instability of CTG expansion size during meiosis and during mitotic divisions, respectively, affecting both the germline and soma [3,14]. Indeed, genetic anticipation can lead to progressively earlier onset of the disease with an increasingly severe phenotype in successive generations [3,13]. On the other hand, somatic mosaicism may contribute to changes in the CTG expansion size, both in cells and tissues in a single affected individual, which can explain the variability of phenotypic expression between different tissues, individuals, as well between family members affected [3,16]. So, additionally to germline instability in the parent, also somatic instability in the parent and offspring may contribute to changes in the CTG expansion size shown with transmission [16]. Congenital DM1, associated with large CTG repeat expansions, is usually transmitted maternally and only rarely paternally [30,31]. Moreover, woman who are considering pregnancy should have adequate counselling and fetal risk of disease should be evaluated by parental repeat size and presence of siblings with DM1 [32].

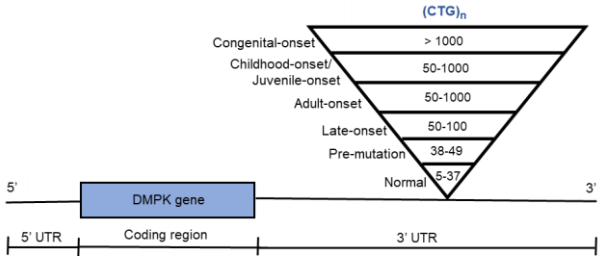


Figure I.1 - Genetic basis of myotonic dystrophy type 1. DM1 is characterized by an unstable CTG repeat expansion at 3'UTR of *DMPK* gene. CTG repeat size correlates with different phenotypes of DM1. (DM1: Myotonic dystrophy type 1; UTR: untranslated region; DMPK: myotonic dystrophy protein kinase). Adapted from Ho *et al.* [22]

1.1.3. DM1 pathogenic mechanisms

In DM1, the mutation located at 3'UTR of the *DMPK* gene involves an expansion of CTG (microsatellite motif) repeats that are transcribed into RNA but not translated, changing its normal translocation from the nucleus to the cell cytoplasm. Therefore, mutant RNA accumulates in the nucleus where it forms aggregates termed nuclear foci. These intranuclear RNA foci trap specific RNA binding proteins that mainly act as splicing regulatory factors, leading to its dysregulation [33,34]. Additional molecular mechanisms of DM1 can be an increased RAN translation [35] and the defective myonuclear positioning which is related to some nuclear envelope proteins mutations [36–39]. Dysregulation of microRNA has also been appointed, but requires further investigation [40]. Another considered hypothesis can be the CTG repeat expansion altering chromatin modeling and organization in the DM1 locus, affecting the expression of neighboring genes: *SIX5* (previously known as DM associated homeodomain protein - DMAHP) and *DMWD*, located immediately downstream and upstream of the *DMPK* gene, respectively [41,42]. However, loss of *SIX5* and *DMWD* does not fully explain the broad features seen in myotonic dystrophy patients, since shown only some features of the disease phenotype in knock-out mice [42,43]. Also, haploinsufficiency of *DMPK* is not considered a major factor in pathogenesis, since *DMPK* knock-out mice reproduce a late-onset myopathy, without a multisystem phenotype [44].

- **RNA toxic gain-of-function**

DM1 molecular pathogenesis is associated with the gene mutation located at 3'UTR of the *DMPK* gene, characterized by an expansion of CTG repeats that are transcribed into RNA but not translated [34]. Therefore, the resulting CUG repeats accumulates in the nucleus, leading to formation of aggregates termed nuclear foci (hairpin structures) which can bind and sequester RNA-binding proteins/splice regulation proteins [45,46]. The most prominent RNA-binding proteins affected by toxic nuclear foci include the CUG RNA binding protein (CUG-BP) family (also known as CELF) and the muscleblind like splicing regulator protein (MBNL) family, that plays important roles in RNA splicing, stabilization, and translation [47,48].

CUG-RNA binding protein 1 (CUG-BP1), a member of the CUG-BP family, displays a high affinity for the expanded CUG repeats, presented at 3'UTR of *DMPK* mRNA [47]. Various studies demonstrate that the transgenic expression of expanded

CUG repeats results in a marked increase of CUG-BP1 levels by inappropriate activation of protein kinases (PKCα and PKCβII), exhibiting a DM1-like muscle phenotype [49,50]. This novel finding indicates the possibility that aberrant activity of protein kinases, triggered by the mutant *DMPK* transcripts, cause hyperphosphorylation and stabilization of CUG-BP1, contributing to some of the phenotypes in DM1 [16]. This increased activity of CUG-BP1 have been linked to unusual alternative splicing of several pre-mRNA transcripts, including cardiac troponin T (cTNT) [51], insulin receptor (IR) [52] and muscle chloride channel (ClC-1) [53,54], which can explain diverse multisystemic effects of DM1 such as cardiac abnormalities, insulin resistance with predisposition to diabetes and myotonia, respectively. Moreover, CUG-BP1 hyperphosphorylation has been noted to inhibit myoblast differentiation and form stress granules which reduce DNA repair [55].

According to various studies, MBNL proteins (MBNL1, MBNL2 and MBNL3 isoforms) were found to bind specifically with the hairpin structures adopted by the expanded CUG-RNA. Indeed, MBNL proteins are sequestered in the nucleus and its normal function is altered, compromising the alternative splicing of diverse pre-mRNAs in various tissues, which can be responsible for the phenotypes presented in DM1 [46,48,56]. The studies using *Mbnl1*-deficient mice have shown that the loss of MBNL function cause aberrant splicing of chloride channel 1 (ClC-1), troponin T2 (TNNT2) and troponin T3 (TNNT3), resulting in DM1-like muscle phenotype [57]. Current experimental evidence suggests that in DM1 patient brain, the cortical and subcortical neurons show the presence of many CUG-RNA nuclear foci. In addition, sequestration of MBNL proteins and deregulated alternate splicing of the N-methyl-D-aspartate (NMDA) receptor, NR1 subunit, amyloid precursor protein (APP) and tau protein are also described [16,58]. Abnormal isoforms of tau protein have been shown in transgenic mice expressing *DMPK* with expanded CUG-RNA repeats, suggesting that CUG-RNA-mediated aberrant splicing can play a significant role in the CNS anomalies described in DM1 [59,60].

Furthermore, have been considered that longer CUG repeats with a higher propensity to form stable hairpin structures show a stronger affinity for MBNL proteins, whereas the shorter CUG repeat tracts tend to remain primarily single-stranded and show a stronger affinity for CUG-BP1. However, further investigation is required, since the possible coexistence of both CUG-BP1 and MBNL RNA complexes can be considered in DM1 [47,61–64]. Additionally, the splicing regulations mediated by the MBNL proteins and CUG-BP1 may be considered antagonistic, with MBNLs favoring adult isoforms and CUG-BP1 promoting the retention of the embryonic isoforms of several of the transcripts found to be misregulated in DM1 [16,65]. The RNA toxic gain-

of-function is currently considered the most acceptable hypothesis that can explain the pathophysiology of DM1. Thus, additional research is necessary to establish the underlying molecular mechanism by which expanded CUG-RNA decrease MBNL activity (abnormal sequestration) and increase CUG-BP1 activity (abnormal stabilization) (presented in Figure I.2), since these RNA targets are alternatively spliced, resulting in altered protein isoforms which can be responsible for the multisystemic effects presented in DM1 [16].

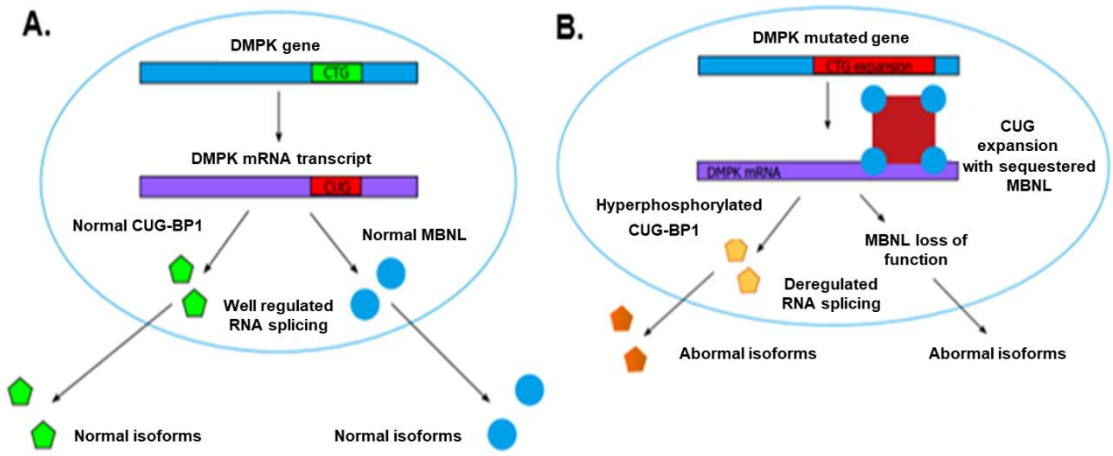


Figure I.2 - RNA toxic gain-of-function in myotonic dystrophy type 1. The normal actions of MBNL and CUG-BP1 in regulating alternative splicing within a cell and pathogenic mechanisms involving MBNL and CUG-BP1, which results in deregulated alternative splicing of RNA targets, were represented. **(A)** Effects of normal CTG repeats at *DMPK* gene. **(B)** Effects of expanded CTG repeats at *DMPK* gene. DMPK: myotonic dystrophy protein kinase; MBNL: muscleblind like splicing regulator protein; CUG-BP1: CUG binding protein 1. Adapted from Ho *et al.* [22]

- **RAN translation**

RAN translation (also termed repeat-associated non-AUG translation) is characterized by no following the traditional rules of RNA translation, as this process can occur without an AUG start codon present and proceeds bidirectionally [66]. It was first reported in DM1 and spinocerebellar ataxia type 8 in 2011, and have been established that RAN translation across CUG-RNA expansion transcripts results in the accumulation of DM1 polyglutamine expansion proteins (polyQn) in DM1 mouse models and human tissues as toxic nuclear aggregates [35]. This process have been associated with patients expressing genetic anticipation and worsened DM1 prognosis, demonstrating

that the DM1 disease mechanism could be related to a combination of RNA toxic gain-of-function and toxicity of RAN proteins [35,66,67].

- **NE contribution to DM1**

The muscle diseases, including DM1, are a group of diseases characterized by muscle weakness and impaired muscle function, which present as common feature the mispositioning of myonuclei [36]. In unaffected individuals, myonuclei remain spaced throughout the periphery of the muscle cell and in affected patients, the nuclei remain within the center of the muscle cell [36,68,69]. Indeed, central nuclei have been used as a pathological marker for differentiating muscle disorders from neurological disorders. On the other hand, have also been show that centrally positioned nuclei can be considered as a tool to determine which myofibers are responding to disease, as a mechanism repair, since it has been proposed that centrally positioned nuclei are a consequence of continual myofiber repair in patients with muscle disease [36,70]. Further, patients with different muscle disorders, including Duchenne Muscular Dystrophy (DMD) and Emery-Dreifuss Muscular Dystrophy (EDMD) show nuclei within the center of individual muscle fibers in muscle biopsies [36,71]. Thus, the study of the mechanisms that drive these nuclear movements and its biological significance is essential to fully understand muscle disease pathogenesis.

Moreover, proteins located in the outer nuclear envelope (ONM), which regulate the interactions between the nucleus and the cytoskeleton, have been identified as mutated in patients with muscle diseases, compromising the nuclear movements and positioning events since the action of the actin cytoskeleton and the ONM proteins is required for a proper movement mechanism [37–39]. Also, the *LMNA* and *EMD* genes, encoding proteins of the nuclear envelope (NE), have been linked to muscle diseases [72]. Additional studies showed an impaired nuclear structure in fibroblasts of DM1 patients, with altered shape of nuclei, altered localization of nuclear envelope proteins and increased nuclear size. Indeed, NE can be considered a novel feature of DM1 and requires further investigation [73].

1.2. The Nuclear Envelope

The Nuclear Envelope (NE), a hallmark of eukaryotic cells, is a highly regulated double membrane that separates the nucleus from the cytoplasm and encloses the nuclear genome [74]. The NE represents the interface between the nucleus and cytoplasm, playing a central role in defining the biochemical identities of each of them, since can separate the genetic material in the nucleus from the cytoplasm and contribute to gene expression regulation [75–78]. The NE is composed by a double nuclear membrane, the nuclear lamina and the nuclear pore complexes (NPCs). The double nuclear membrane is composed by two morphologically distinct and interconnected domains, the inner nuclear membrane (INM) and outer nuclear membrane (ONM) (Figures I.3-4) [79–83]. These membranes are separated by the perinuclear space (PNS) which corresponds to a 30 to 50nm gap and is continuous with the lumen of the endoplasmic reticulum (ER) [84].

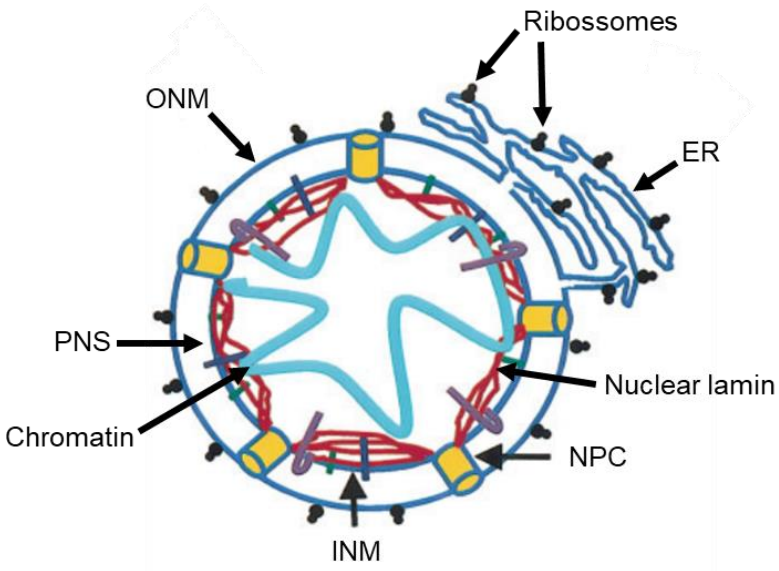


Figure I.3 - Schematic diagram of the nuclear envelope. The nuclear envelope consists of inner and outer nuclear membranes that are separated by the perinuclear space. The nuclear lamina interacts with chromatin. Representative inner nuclear membrane proteins, emerin (green), LAP2 (dark blue), and MAN1 (purple), are shown. (INM: inner nuclear membrane; ONM: outer nuclear membrane; PNS: perinuclear space; NPC: nuclear pore complex; ER: endoplasmic reticulum). Adapted from Östlund *et al.* [82]

The nuclear envelope contains a network of interacting proteins (Figure I.4), which are essential components of the cell, regulating several nuclear functions, described below.

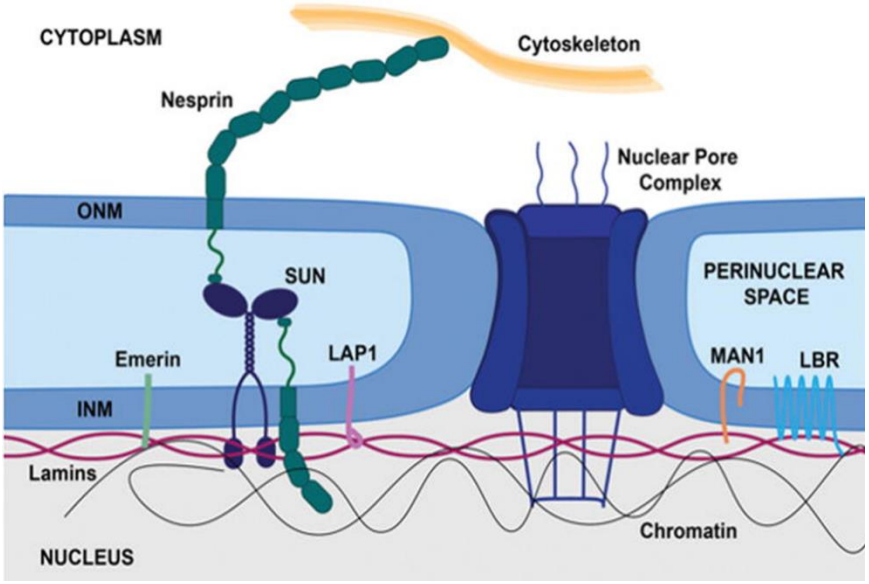


Figure I.4 - Topology of the nuclear envelope. Inner and outer nuclear membranes (INM and ONM, respectively) are separated by the perinuclear space (PNS). The nuclear lamina, composed by lamin proteins, interacts with INM proteins and chromatin. Presented INM proteins (emerin, SUN, LAP1, MAN1 and LBR) can interact with lamins and underlying chromatin. Presented ONM proteins (nesprins) provide a connection from the nucleus to the cytoskeleton. Taken from Puckelwartz *et al.* [83]

1.2.1. Inner nuclear membrane (INM)

The INM encompasses many integral membrane-associated proteins, including lamin B receptor (LBR), MAN1, emerin, lamin associated proteins (LAP1 and LAP2) and SUN1 (Sad1/UNC-84) [74,84,85] that are characterized by maintaining close associations with the nuclear lamina and/or underlying chromatin [86]. These associations are involved in processes like genome regulation, inheritance and protection [86,87]. In addition, a shared motif common to the nucleoplasmic domains of the INM proteins LAP2, emerin and MAN1, called LEM domain, binds to Barrier to Autointegration Factor (BAF), a chromatin binding protein [74,88]. SUN1 belongs to the LINC complex (Linker of Nucleoskeleton and Cytoskeleton) that is described below.

- **Lamin B Receptor**

Lamin B receptor (LBR) is an INM integral protein, containing a hydrophilic N-terminal end protruding into the nucleoplasm, a nucleoplasmic C-terminal tail and eight hydrophobic segments that span the membrane [89]. The N-terminal domain of LBR tethers heterochromatin to the nuclear periphery at early developmental stages, contributing to the shape of interphase nuclear architecture [89,90]. Also, LBR transmembrane domain exhibits sterol reductase activity and has been proposed that many cells depend on LBR to produce cholesterol [89]. Mutations in the *LBR* gene cause diverse congenital disorders such as Greenberg skeletal dysplasia and Pelger-Huët anomaly, resulting in sterol reductase dysfunction and defects in cholesterol synthesis [91,92].

- **MAN1**

MAN1 is an INM integral protein associated to beta/bone morphogenic protein (TGFβ/BMP) signalling. This INM protein is a receptor-regulated SMAD cofactor that terminates TGFβ superfamily signals. SMAD proteins are key transcription factors of TGFβ superfamily. Loss of MAN1 have been associated with the development of autosomal dominant diseases (osteopoikilosis, Buschke-Ollendorff syndrome and melorheostosis), characterized by an increase in bone density [93].

- **Emerin**

Emerin is an INM protein, encoded by the *EMD* gene and composed of a 220 amino acid N-terminal nucleoplasmic domain, a 23 amino acid C-terminal transmembrane domain and an 11 residue lumenal domain. Newly synthesized emerin is inserted into the endoplasmic reticulum (ER) post-translationally, diffusing throughout the ER into the connecting membranes of the NE and once inside the nucleus, for proper localization at the NE, binds to lamins [94,95]. Emerin binds to lamin-A and lamin-B types via its LEM domain, and to BAF protein [96,97], which is a conserved chromatin protein essential for cell division, recruiting emerin to chromatin, and regulates higher-order chromatin structure during nuclear assembly [98,99]. Emerin is ubiquitously expressed [100–102] and can interact with structural proteins, including nuclear actin, nesprins, SUN proteins, lamin A/C, lamin B, and LAP1, which are predicted to be key regulators of nuclear architecture. Indeed, emerin has been implicated in the regulation of nuclear and genomic architecture, cell signaling and gene expression [103–

106]. The X-linked form of Emery-Dreifuss muscular dystrophy (X-EDMD) is caused by a significant reduction or absence of emerin and was the first human disease found as a NE-specific defect muscle weakening [107,108]. Previous studies with emerin null fibroblasts demonstrate ineffective nucleus-cytoskeleton interactions, and that the *EMD* gene is necessary to maintain the structure of individual nuclei and the position of nuclei [36,109,110]. Additional studies show that emerin-null cells have decreased elasticity and increased nuclear fragility, contributing to the nuclear defects observed in EDMD patient cells, since emerin plays important roles in maintaining nuclear architecture and is implicated in muscle disease [36,111,112].

- **Lamina-associated proteins (LAP1 and LAP2)**

Lamina-associated polypeptide 1 (LAP1), encoded by the *TOR1AIP1* gene, is a ubiquitously expressed type II transmembrane protein comprising a nucleoplasmic N-terminal domain, a single TM domain and a lumenal C-terminal domain, located in the perinuclear space of the NE [113]. This protein was first identified as three antigenically related polypeptides in rat liver NE extracts [114]. The rat and mouse isoforms were designated LAP1A, LAP1B, and LAP1C (molecular weights of 75, 68 and 55kDa, respectively), differing only in the nucleoplasmic region [114,115]. Also, two functional LAP1 isoforms, LAP1B and LAP1C, are actually known in humans (molecular weights of 68 and 55 kDa, respectively) [116,117]. The function of LAP1 remains poorly understood, however its interaction with NE proteins and chromosomes has been reported [118]. Since LAP1 can interact with lamins, may be involved in the positioning of lamins and chromatin in the NE, contributing to the chromatin regulation, nuclear architecture preservation and, therefore, maintenance of the NE structure [113,119]. Also, LAP1 and emerin interaction, which is associated with the X-linked Emery-Dreifuss muscular dystrophy disorder [120], have been appointed to be involved in the skeletal muscle preservation and NE integrity [121]. Since LAP1 has been reported to interact with torsinA in the NE [122], is also known as torsinA interacting protein 1 (TOR1AIP1). In this way, LAP1 is associated to NE dynamics and is a crucial component for torsinA ATPase activity [123,124]. A mutation of a glutamic acid within torsinA is responsible for the most cases of early-onset torsion dystonia (DYT1 dystonia), which is a movement and neurological disorder [125]. LAP1 is considered a crucial factor in the production of healthy sperm cells [126] and human LAP1B can bind to protein phosphatase 1 (PP1) in the nucleoplasm, resulting in its dephosphorylation [127]. Also, have been shown that conditional deletion of LAP1 from mouse muscle causes muscular dystrophy leading to

early lethality. In this way, LAP1 can be associated with muscular diseases since the presence of this protein is necessary for proper muscular functioning [117,121].

Lamina-associated polypeptide 2 (LAP2) is composed by a family of six alternatively spliced proteins, containing LAP2 β, γ, δ and ε type II transmembrane proteins [128]. LAP2α localizes to the nucleoplasm and interacts with chromatin through its N-terminal and C-terminal domain. The latter also binds to A-type lamins and retinoblastoma (Rb) protein, regulating cell cycle progression through retinoblastoma-mediated E2F-dependent transcription. LAP2α can regulate the differentiation of adult tissue progenitor cells [129,130]. Therefore, this protein may be associated to diverse muscle disorders, including Emery-Dreifuss and Limb-girdle muscular dystrophy 1B, which present childhood and early adulthood onset characterized by a fault in stem cell-mediated adult tissue homeostasis [129,131]. On the other hand, LAP2β can interact with chromatin, lamin B *in vitro* and *in vivo* [115,128,132].

1.2.2. Outer nuclear membrane (ONM)

The ONM is biochemically and functionally similar to the endoplasmic reticulum (ER) and can be considered as its sub-compartment involved in protein synthesis [74,128,133]. ONM is composed by nesprin proteins (nesprin1, nesprin2 and nesprin3) that can provide attachment sites for structural elements of the cytoplasm [134–137]. Nesprins belongs to the LINC complex (Linker of Nucleoskeleton and Cytoskeleton) that are described below.

1.2.3. Nuclear Lamina

The nuclear lamina, a thin matrix of ~10-20nm of lamins, representing one - type V - of six subtypes of the intermediate filaments (IF) superfamily, and are constituted by A- and B-type lamins, which differs in protein features and expression patterns [138,139]. The nuclear lamins guarantees the integrity and supports the nuclear structure [80,114] and are linked to INM proteins, establishing a connection between the cytoskeleton, the nucleoskeleton and the genome [140,141]. Since the nuclear lamins contact directly with chromatin, might play a role in diverse cellular processes, including gene expression, DNA replication and repair, chromosome organization, transcriptional control and heterochromatin maintenance [74,85,142].

- **Lamins**

The nuclear lamina is composed by lamin proteins which have a close association with INM proteins and, therefore, can play an essential role in the nuclear architecture [143]. A- and B-type lamins are the main elements of the nuclear lamina in mammalian cells [144]. The A-type lamins are encoded by the *LMNA* gene which gives rise to lamin A and lamin C, C2 and C3 isoforms by alternative splicing [145]. On the other hand, the B-type lamins are encoded by the *LMNB1* gene (lamin B1) and *LMNB2* gene (lamins B2 and B3 isoforms) [145]. Mutations in the lamin genes cause distinct heritable diseases collectively termed as laminopathies. However, most of these mutations are in the *LMNA* gene, affecting distinct tissues and give rise to a range of rare and genetic disorders, including autosomal dominant form of Emery-Dreifuss muscular dystrophy (A-EDMD), Limb–Girdle muscular dystrophy type 1B (LGMD1B) and dilated cardiomyopathy with conduction-system disease (DCM-CD). Lamins provide both structural support for the nucleus and sites of attachment for chromatin, and defects in these functions may contribute to disease pathogenesis [84,146–148]. The expression of lamin A/C is tissue-specific and developmentally regulated, whereas the lamin B protein is ubiquitously expressed in all mammalian tissues. Lamins A/C are expressed only in differentiated cells, but C2 isoform is uniquely expressed in germ cells [149].

The first study using A-type lamin-deficient mices (lmna -/-), in 1999, showed changes in nuclear structure and localization [150]. These characteristics were also observed in human *EDMD* patients carrying *LMNA* mutations [151]. Both *EMD* and *LMNA* genes are necessary to nuclei position, to maintain the structure of the individual nuclei, and to maintain proper gene regulation. Mutations in emerin and lamin A/C encoding genes compromises the latter processes and are associated to muscle disease [36,72]. Additional studies showed that in the absence of lamin A/C, the ability of the nucleus to support physical stress [112,152], of the cell to organize its genome [153,154] and nuclear movement are compromised [155–158]. Further, experiments in *Drosophila* larval muscles demonstrate a dramatically mispositioned nuclei when lamin C is absent, making clear that lamin A/C is essential for a proper nuclear positioning in muscle cells. Thus, the role of lamin A/C in nuclei positioning may contribute to muscle weakness and disease [159]. Additionally, mutations in lamin A/C have been shown to inhibit the ability of the nuclear lamin to anchor the LINC Complex, compromising the nuclear movement and can be related to muscle disease pathogenesis [158].

Like all IF proteins, lamins have a tripartite structure consisting of a long α-helical domain flanked by globular amino-terminal (head) and carboxy-terminal (tail) domains

(Figure I.4). Indeed, lamins are constituted by a globular N-terminal head domain, a coiled-coil central rod domain also termed central α-helical domain, and a C-terminal tail domain. Their unique features are a nuclear localization signal (NLS), an immunoglobulin (Ig)-fold domain, and a CaaX motif (C: cysteine; a: aliphatic residue; X: any residue) at the tail domain (Figure I.5) [160]. These proteins suffer an extended process of post-translational modifications (PTMs), since the mammalian lamins B1, B2, and A are synthesized as prelamins containing a CaaX motif at their carboxy-terminal tails (C-terminal) in which the cysteine is post-translationally modified by farnesylation and methyl esterification (Figure I.5). Indeed, the CaaX motif undergoes sequential processing, which begins with an addition of the hydrophobic farnesyl group to the cysteine residue and continues with cleavage of the last three amino acids by FACE1 or Zmpste24 metallopeptidases. Then, a methyl group is added to the exposed cysteine by isoprenylcysteine carboxyl methyltransferase. Only lamin A undergoes an additional cleavage of 15 amino acids upstream of the farnesylated and methylated cysteine by Zmpste24. Therefore, whereas B-type lamins remain permanently farnesylated, mature lamin A undergoes further proteolytic cleavage which removes the modified C-terminal cysteine together with an additional 15 amino acids. Further, mutations in either the *LMNA* or *ZMPSTE24* genes that fail to remove the farnesylated and methylated cysteine result in severe diseases such as Hutchinson–Gilford progeria syndrome (HGPS) [161–163].

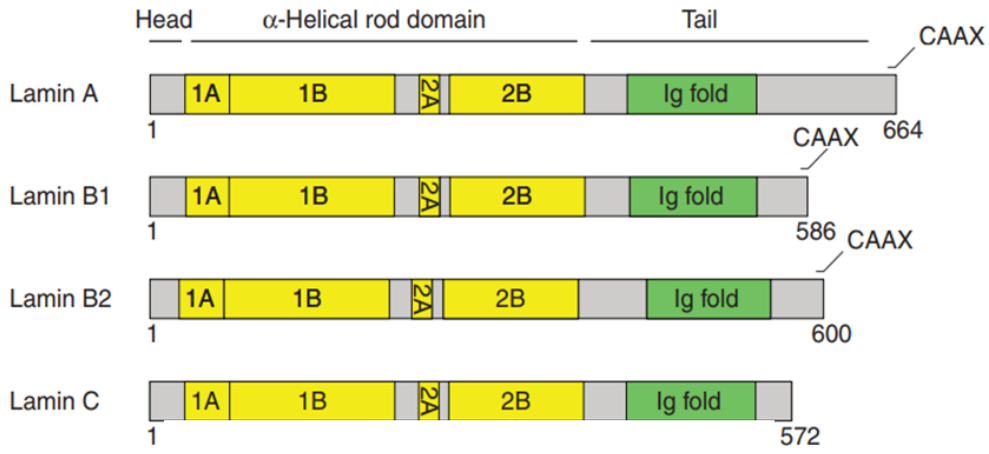


Figure I.5 - Schematic representation of the nuclear lamins structure. Nuclear lamins have a tripartite structure. The rod domain or central α-helical domain comprises four α-helical segments termed 1A, 1B, 2A and 2B. Each α-helical segment is connected by short intervening subdomains termed L1, L12 and L2. The carboxy-terminal tail domain contains a nuclear localization signal, an Ig domain and a CaaX box. (Ig: immunoglobulin). Adapted from Dittmer *et al.* [161]

1.2.4. Nuclear pore complexes (NPCs)

The nuclear pore complex (NPC) is an eightfold symmetrical structure constituted by aqueous multiprotein channels that can penetrate the ONM and INM membranes, regulating the bidirectional exchange of macromolecules, including proteins, RNA, and ribonucleoprotein complexes, between the nucleus and cytoplasm. Indeed, the principal functions of the NPC are the facilitation of nucleocytoplasmic transport and generate a diffusion barrier to separate the cytoplasm from the nuclear compartment [164–166]. The vertebrate NPC is constituted by a central scaffold measuring 125 nm in diameter and 70 nm in height, eight cytoplasmic filaments and eight nuclear filaments. The central scaffold contains a large channel with 50 nm in diameter and is composed of a cytoplasmic and a nuclear ring (Figure I.6) [167–170]. The NPCs are built from about ~30 different proteins, the nucleoporins - NUPs. Each NUP occurs in eight or multiples of eight to yield a total of ~600 peptides per NPC [171–173]. Ions and small molecules diffuse freely through the nuclear pore while larger molecules, over 40–60 kDa in size, require active translocation by transport receptors and specific transport signals, namely, nuclear localization signal (NLS) or nuclear export signal (NES) [174]. Soluble transport receptor proteins (importins and exportins) of the karyopherin (kap) family, recognize NLSs or NESs directly, by β-karyopherins (β-kaps), or indirectly through an adaptor karyopherin termed α-kap. This karyopherin macromolecule binding mediates nucleocytoplasmic transport [175]. Indeed, a nuclear localization sequence and a nuclear export sequence is responsible for import and export, respectively. Ran, a small GTPase that is a key component for the generation of the directionality of nucleocytoplasmic transport, can adopt two distinct conformations that depend on the bound nucleotide (GTP or GDP). So, the directionality of transport is governed by a concentration gradient of Ran-GTP, which is sustained at a high level inside the nucleus and at a low level in the cytoplasm [166,176–178]. Ran-GTP not only disassembles import complexes upon entrance into the nucleus, but also promotes the assembly of export complexes inside the nucleus [176–181]. Furthermore, the diffusion barrier is formed by extended nucleoporin segments that contain numerous phenylalanine-glycine (FG) repeats [182–185], which serve as docking sites for karyopherins, that transport the cargo through the permeability barrier [176–181].

The NPC and its components are also involved in other cellular functions, including chromatin organization, regulation of gene expression and cell division [186,187,196,188–195]. Also, diverse proteins have been related with these multiprotein

channels, namely, cell proliferation regulators, transcription factors, translation initiation factors, RNA binding proteins and hormone receptors [164,197–199].

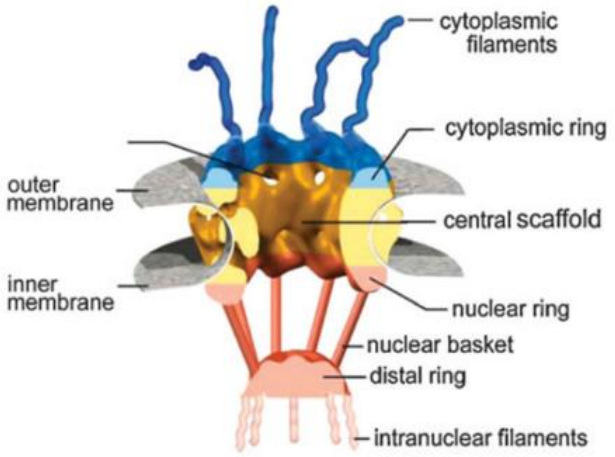


Figure I.6 - Schematic representation of the nuclear pore complex structure. The nuclear pore complex is constituted by a central scaffold, composed of a cytoplasmic and a nuclear ring. There are eight cytoplasmic filaments and eight intranuclear filaments. The nuclear basket allows the nuclear ring and distal ring communication. Taken from Peters *et al.* [170]

- **Nucleoporins**

The nucleoporins (NUPs) can be separated according to their localization in the NPC and normally are named with a number that agrees to their molecular weight. However, since the molecular weight varies in different species, a uniform nomenclature for nucleoporins does not exist. So, based on their approximate localization within the NPC, the nucleoporins can be classified into six categories: integral membrane proteins of the pore membrane domain of the nuclear envelope (POMs) nucleoporins, membrane coat nucleoporins, adaptor nucleoporins, channel nucleoporins, nuclear basket nucleoporins, and cytoplasmic filament nucleoporins (Figure I.7; Table I.3) [200]. Nucleoporins can be constituted by α-helical regions, β-propellers, and phenylalanine-glycine (FG) repeats structural units [201].

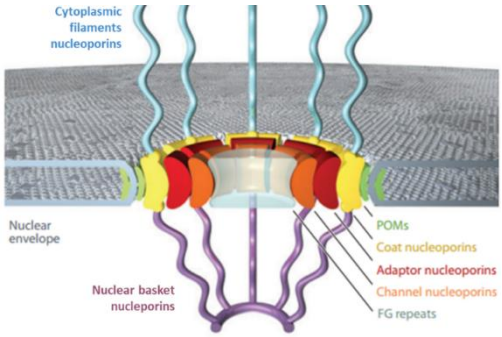


Figure I.7 – Schematic representation of the nucleoporin categories. The nuclear pore complexes are constituted by ~30 nucleoporins, which can be divided according to their localization in the NPC: pore membrane domain of the nuclear envelope (POMs) nucleoporins, coat nucleoporins, adaptor nucleoporins, channel nucleoporins, nuclear basket nucleoporins, and cytoplasmic filaments nucleoporins. Adapted from Hoelz *et al.* [166]

Table I.3 - Classes of nucleoporins. (POMs: proteins of the pore membrane domain of the nuclear envelope; NUPs: nucleoporins). Adapted from Hoelz *et al.* [166]

POMs nucleoporins	**Coat NUPs**	**Adaptor NUPs**	**Channel NUPs**	**Cytoplasmic filaments NUPs**	**Nuclear basket NUPS**
NUP210 NDC1 POM121	NUP160 NUP133 NUP107 NUP96 NUP85 SEH1L NUP43 NUP37 SEC13 ELYS	NUP205 NUP188 NUP155 NUP93 NUP35	NUP62 NUPL1 NUP45 NUP54	RANBP2 NUP214 NUP98 NUP88 ALADIN NUPL2 RAE1	NUP153 TPR NUP50

In addition to nucleocytoplasmic transport, NUPs are involved in other cellular processes such as gene expression, chromatin organization, differentiation, epigenetic regulation, DNA damage repair and mitosis [166,174,202]. Thus, genetic alterations in many *NUP* genes are linked to cellular defects and to various human diseases, including neurological and cardiovascular disorders, autoimmune dysfunctions, and cancer [166,203]. Also, mutations in either *NUP133* or *NUP120* genes result in clustering of

NPCs into one or a few aggregates in the NE, suggesting a role in NPC-NE organization [204].

1.3. The LINC Complex

The LINC complex (Linker of Nucleoskeleton and Cytoskeleton) is a structure that spans the NE to link the nucleoskeleton and cytoskeleton. ONM KASH proteins and INM SUN proteins interact to form the core of the LINC complex (Figure I.8). SUN proteins provide a connection to nuclear structures while acting as a tether for KASH proteins, which contain binding sites for cytoskeletal components [205]. Starr and Han first recognized the role of the LINC complex in nuclear positioning and anchoring in *Caenorhabditis elegans* [136,206]. They showed that a large actin-binding protein, KASH protein ANC-1, was required for tethering hypodermal cell nuclei. Localization of ANC-1 to the ONM was found to be dependent of the SUN protein UNC-84, as the latter together with ANC-1 formed a protein bridge that crossed the NE and coupled the nuclear lamina to actin filaments [136,206]. The LINC Complex is required for nucleus-cytoskeleton interaction and provides the force to move nuclei, which is essential for a proper nuclear movement and positioning in muscle cells [84]. Several studies show that the LINC complex has an important role for diverse cellular functions, since is implicated in cytoskeletal organization [137], cell division [207] and organelle positioning [208]. Additional functions include cell polarity, and potentially mechanotransduction [209].

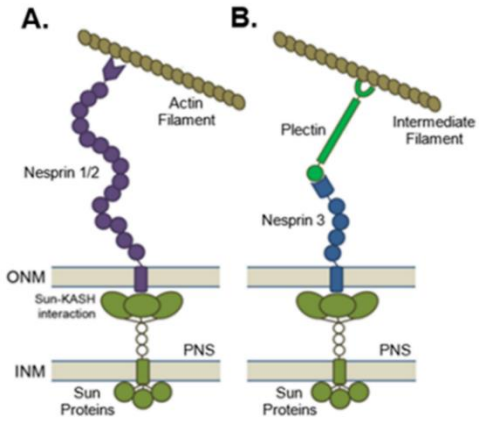


Figure I.8 - Schematic overview of SUN-KASH interactions. LINC complexes are composed by the Sad1/UNC-84 (SUN) INM proteins and Klarsicht/ANC-1/SYNE homology (KASH) ONM proteins. SUN domain proteins form a binding pocket for KASH domain proteins in the perinuclear space. **(A)** KASH proteins (nesprin 1/2) and cytoskeletal protein (actin) direct interaction. **(B)** KASH protein (nesprin 3) and cytoskeletal protein (intermediate filament) interaction via cyto-linker protein (plectin). (ONM: outer nuclear membrane; INM: inner nuclear membrane; PNS: perinuclear space). Adapted from Kim *et al.* [209]

- **SUN Proteins**

The Sad1/Unc-84 (SUN) proteins, specifically located at INM, are type II transmembrane proteins. The N-terminal region of this proteins is exposed to the nuclear contents, while C-terminal sequence, consisting of a coiled-coil region, extends into the PNS [140,210]. There are five conventional SUN proteins in mammals, however only two of these (SUN1 and SUN2) are widely expressed [211]. SUN1 has been described as necessary for DNA double-strand break repairs, pairing meiotic chromosomes, taking part in RNA export as a messenger and nucleolar morphogenesis, while SUN2 is responsible for the formation of actin cables that are able to move the nucleus [211]. Recent studies have revealed the involvement of SUN1 and SUN2 in the DNA damage response (DDR), as SUN1 and SUN2 double knockout fibroblasts exhibit DNA damage and compromised DDR activation [212]. Have been demonstrated that *SUN1* and *SUN2* mutations disrupt connections between the nucleus and the structural elements of cytoplasm, leading to a defective myonuclear positioning by clustering of nuclei in muscle cells. In this way, SUN1 and SUN2 proteins are essential components of the LINC Complex since can be involved in nuclear positioning and cell migration. Also, can be associated to loss of myonuclear anchoring and impaired muscle function, playing a central role in muscle disease pathogenesis such as Emery–Dreifuss muscular dystrophy (EDMD) [213]. On the other hand, have been shown that mutations in the *EMD* and *TMPO* gene (coding genes of emerin and LAP2α, respectively) in combination with mutations in *SUN1*, can affect both cellular properties and expression levels of NE proteins. SUN1 protein, such as crucial component of the LINC complex, may play a key role in the positioning of muscle cells by anchoring interactions at the NE and can be associated to muscular dystrophies such as EDMD [214]. Defects in SUN proteins might lead to the progression of laminopathies since its nucleoplasmic domains interact with lamins, and, therefore, can play significant roles in diverse cellular functions, such as regulation of the intracellular position of the nucleus [215]. Also, have been reported that, together with SUN proteins, can be formed a complex at the INM, composed by lamin A, emerin, and short nesprin isoforms interactions. This network of interactions is seen to be critical for maintaining a proper nucleoskeleton-cytoskeleton interaction and nuclear integrity, so it can be associated with the correct positioning of nuclei in muscle cells, playing an important role in muscle disease [216].

- **KASH Proteins**

The Klarsicht/ANC-1/SYNE homology (KASH) proteins (also known as nesprins, nuclear envelope spectrin repeat proteins), specifically located at ONM, are type II transmembrane proteins, characterized by their cytoplasmic N-terminal region anchored to the ONM interacting with the cytoskeleton and other cytoplasmic proteins, while their C-terminal sequence containing an highly conserved region of 35 aa is rooted in the PNS. The KASH proteins engage directly with available SUN domains through this short peptide. In this way, the LINC complex, spanning both nuclear membranes, is able to mechanically couple nuclear and cytoplasmic structures [140,211].

In mammals there are six encoded KASH proteins, namely nesprins 1-4, lymphoid-restricted membrane protein and KASH5. The members of these protein families are expressed differently in various tissues due to alternative splicing [140,211,217]. Two of these, nesprin1 and nesprin2, are encoded by a pair of complex genes and the largest isoforms, nesprin1-Giant and nesprin2-Giant, have masses of about 1000 kDa and ~800 kDa, respectively [218,219]. Given their size, functional domains and splicing isoforms, nesprin1 and nesprin2 can interact with diverse proteins and have been associated with many diseases like myopathies, neurological disorders, cancer, hearing loss, arthrogryposis, EDMD and EDMD-like phenotypes. Moreover, have been demonstrated that lamin A/C mutations affect the LINC complex, altering nesprin1, nesprin2 and SUN2 localization [83,220]. Nesprins are also implicated in mRNA export, opening the possibility that these proteins help anchoring SUN1 close to the NPC aiding and enabling it to accomplish its role in mRNA export [221]. Recent studies also indicate that forces transmitted via nesprin1 in isolated nuclei led to changes in nuclear stiffness, resulting in the phosphorylation of emerin [222]. Moreover, nesprin1 and nesprin2, upon mechanical stimulation, can induce altered nuclei positioning, shape, and gene expression of biomechanical response genes. These observations suggest that both nesprin1 and nesprin2 play important roles in regulating gene expression and sensing mechanical force [223]. The third member of the family is nesprin3 which binds to plectin, a cyto-linker protein that provides a connection between the NE and the intermediate filament system. So, whereas nesprin1 and nesprin2 connect the NE to microfilaments, nesprin3 may function as a link between the NE and the cytoplasmic intermediate filament network [224,225]. Indeed, the loss of nesprin3 in zebrafish and mice demonstrates a reduced association between the intermediate filament system and the NE [224,226]. Nesprin4 and KASH5 function as NE adaptors for kinesin-1 and cytoplasmic dynein, respectively [227,228].

CHAPTER II: Aims of thesis

DM1 is a multisystemic, dominant autosomal disease characterized mainly by myotonia (delayed relaxation after skeletal muscle contraction), progressive muscle weakness, accompanied by progressive muscle wasting and several multisystemic features, including cardiac conduction defects, development of cataracts and impaired respiratory and gastrointestinal functions [13,229–231]. DM1 is caused by expansion of CTG trinucleotides repeats in the 3′ untranslated region (UTR) of the *DMPK* gene located at the chromosome 19q13.3 [13]. Disease severity and age of onset correlate with repeat length, and repeat expansions of >1000 often result in a severe congenital form of DM1 [232]. Interestingly, the muscle diseases, including DM1, are a group of diseases characterized by muscle weakness and impaired muscle function, which present as common feature the mispositioning of myonuclei [36]. The myonuclei remain spaced throughout the periphery of the muscle cell, in unaffected individuals, and in patients the myonuclei are often clustered in the centre of muscle cell [36,68,69]. Indeed, central nuclei have been used as a pathological marker for differentiating muscle disorders from neurological disorders [70]. Further, muscle biopsies from patients with different muscle disorders, including Duchenne Muscular Dystrophy (DMD) and Emery-Dreifuss Muscular Dystrophy (EDMD) show nuclei prominently within the centre of individual muscle cells [36,71,233]. Moreover, several NE proteins have been pointed as crucial proteins controlling not only the nuclear positioning and nuclear movements but also muscle functions. Among these are, lamin A/C, emerin, LAP1 and SUN proteins whose important muscle functions have been ascribed. Moreover, mutations on these genes have been associated with muscle diseases given they are crucial components in muscle differentiation and myonuclear movement regulation and consequently muscle function [36,72]. Regarding DM1 only a few studies have indicated that NE structure and function are altered in DM1 patients, suggesting that in this muscle disease the NE might have an important role and requires further investigation [73,234].

Therefore, the main aim of this dissertation is to characterize the NE alterations observed in DM1. For that purpose, we have used as cellular model of disease, human patient fibroblasts. The human patients' fibroblasts included in this study have 1000 CTG and 2000 CTG repeats, representing the adult and congenital DM1 types, respectively. The controls fibroblast presents CTG repeats between 5 and 27 repeats. Further, the spectroscopic analysis of the human patient and control fibroblasts were also performed.

The specific aims of this dissertation are:

1. Perform a FTIR spectroscopy study in DM1 human models;
2. Identify spectral differences in DM1 human models by principal component analysis (PCA);
3. Evaluate the nuclear profile in DM1 human models;
4. Evaluate the subcellular localization of NE proteins in DM1 human models.

CHAPTER III: Spectroscopic analysis of DM1 models

3.1. Introduction

The vibrational spectroscopic techniques are potential tools for biomedical applications since has made remarkable progress in the field of clinical evaluation [235,236]. The infrared spectroscopy is a technique based on the vibrations of the atoms of a molecule. In this way, when the infrared radiation (IR) crosses through a sample, its functional groups can absorb this radiation, creating vibrations that are molecule specific and provide direct information about the biochemical composition of each analyzed sample. The mid-infrared (MIR) part of the spectra, between 4000 - 600 cm^{-1}, is identified as the fundamental region where these vibrations can occur [237]. The obtained infrared absorbance spectrum provides data about the molecules by the resultant wavenumbers and peaks. Therefore, for the spectra analysis, it is essential the use of tables which correlate the frequencies with the experimental spectra and functional groups, since the frequencies obtained are specific for each analysed sample [235,238]. Fourier Transform Infrared Spectroscopy (FTIR) is a rapid, noninvasive and reagent-free metabolomic technique that allows to discriminate the metabolic profile of different biological samples, such as tissues, cells and fluids. This technique examines the biochemical composition and can analyze the functional groups, bonding types, and molecular conformations present in several biomolecules such as proteins, lipids, nucleic acids, amino acids and fatty acids simultaneously, with a minimum amount of sample (micrograms to nanograms). This approach is based on the vibrations of the atoms in a molecule, caused by the interaction of infrared radiation (IR) with the matter [236,237,239–241]. The resulting peaks correspond to the frequencies of vibrations between the bonds of the atoms in the sample [242]. Attenuated total reflectance (ATR) is one of the most used approaches for FTIR sampling, producing high quality and reproducible spectra [239]. Infrared spectroscopy has a high potential for the clinic since it has already used in cell lines, tissues, and blood specimens as biological samples for medical diagnostics for some diseases [241,243–247]. By comparison between pathological and non-pathological samples, this technique can detect biochemical changes presented in diseases, serving as diagnostic tool [235,248]. Indeed, FTIR spectroscopy has already proved to have potential in cancer [245,249–254], leukemia [255], diabetes [246,256], rheumatoid arthritis [257] and Gaucher disease [247] diagnosis. Moreover, has been used to study protein misfolding and aggregation, an hallmark in diverse diseases, including Alzheimer's, Parkinson's and Huntington's disease [258]. To date, no FTIR spectroscopy studies have been performed on muscular dystrophies, including DM1. ATR-FTIR spectroscopy has already been applied in disease diagnosis using different

cell lines, including cultured fibroblasts obtained from Gaucher disease patients [247] and prostate cancer cell lines obtained from affected patients [241].

ATR-FTIR spectroscopy is a suitable screening technique that may allow to identify spectral differences between the cultured fibroblasts derived from healthy and DM1 patients. Therefore, the main aim of this study is to characterize the biomolecular profile of human DM1 models (patients and controls) using ATR-FTIR spectroscopy and multivariate analysis, in order to provide novel molecular insights into DM1 pathology.

3.2. Materials and Methods

3.2.1. Samples

Fibroblasts from biopsies from donors with different CTG repeat sizes are: apparently healthy controls, with a CTG repeat size between 5 and 27 (GM02673), clinically affected with a CTG repeat size of approximately 1000 - DM1 1000 (DM1, GM04647) and clinically affected with a CTG repeat size of approximately 2000 - DM1 2000 (DM1, GM03989) were obtained from Coriell Institute. All the donors were male and Caucasian. The human patient's fibroblasts included in this study have 1000 and 2000 CTG repeat size, representing the classical adult and congenital DM1 subtypes, respectively.

3.2.2. Cell Culture

Fibroblasts were cultured in DMEM (Dulbecco's Modified Eagle Medium, high glucose) (Gibco™) supplemented with 15% FBS (Fetal Bovine Serum, Origin South America) (Gibco™). All the washes performed were made using Dulbecco's 1X PBS (Phosphate Buffered Saline), without Ca^{2+} and Mg^{2+} (BioConcept Ltd. Amimed®). Fibroblasts were sub cultured whenever 85-90% confluence was reached. It was incubated 0.05% Trypsin-EDTA (Gibco™) for 5 min whenever subculture was needed. Cell cultures were maintained at 37ºC under 5% CO_2. Fibroblasts were maintained until passage number 13 was reached.

3.2.3. Preparation of cells for FTIR

Between passage number 11 and 13, after reaching around 95% of confluency, cells were prepared for FTIR analysis. Fibroblasts were detached from the plate using 0.05% Trypsin-EDTA (Gibco™), as descried above, and centrifuged at 1000 rpm for 3

minutes. Following centrifugation, the supernatant was discarded, and cells were resuspended in culture medium. Cells were counted using the Trypan Blue assay. Briefly, to a 90 µL aliquot of cells suspension, 10 µL of 0.4% Trypan Blue were added and incubated for 1 minute at room temperature. The unstained (viable) cells were counted in a hemocytometer, and cell concentration calculated, and a total of $0.9x10^6$ cells were used for each replicate. Cells were centrifuged at 1000 rpm for 3 minutes to remove the medium and resuspended in Dulbecco's 1X PBS. Further, PBS was removed by centrifugation at 1000 rpm for 3 minutes and cell pellets were kept on ice until FTIR analysis.

3.2.4. FTIR Measurements

FTIR spectra were acquired in ATR mode in a FTIR spectrometer (Alpha Platinum ATR, ©Bruker), and pre-processed using the OPUS software (©Bruker). The spectra were obtained in the wavenumber range between 4000 and 600 cm^{-1} (mid-infrared region), with a resolution of 8 cm^{-1} and 64 coadded scans. Spectra acquisition was performed in a room with controlled temperature and relative humidity (24ºC and 40%, respectively). A background spectrum was acquired with the empty crystal before each different cell line. Three technical replicates for control, DM1 1000 and DM1 2000 fibroblasts were used. The cell pellet was pipetted onto the crystal and spectra were acquired after the samples were completely air-dried. Between each measurement the crystal was cleaned with ethanol 70%, followed by distilled water.

3.2.5. FTIR Data Analysis

All spectra were baseline corrected and area normalized (Figure III.1). The normalized spectra were derived, using the second derivative and Savitzky-Golay method with 3 smoothing points, to resolve the obtained peaks and maximize the differences between the spectra. Spectra were processed using The Unscrambler X® software (v.10.4, CAMO, Oslo, Norway). The spectral regions 3000-2800 cm^{-1}, 1800-1500 cm^{-1} and 1200-900 cm^{-1} were chosen for Principal Component Analysis (PCA) of the control, DM1 1000 and DM1 2000 fibroblasts and all spectral assignments were made according to cited literature references.

3.3. Results

In order to investigate the biomolecular profile of human DM1 models (controls and DM1), ATR-FTIR spectroscopy was performed. Briefly, the human DM1 models used were fibroblasts from donors with a CTG repeat size of 5 to 27 (healthy), a CTG repeat size of approximately 1000 (DM1 1000) and a CTG repeat size of approximately 2000 (DM1 2000). These fibroblasts were used between passages number 11 and 13 for the obtaining baseline-corrected and area normalized spectra (Figure III.1) and then specific spectra for different regions: 3000-2800 cm^{-1}, 1800-1500 cm^{1} and 1200-900 cm^{-1} (Figure III.2-4).

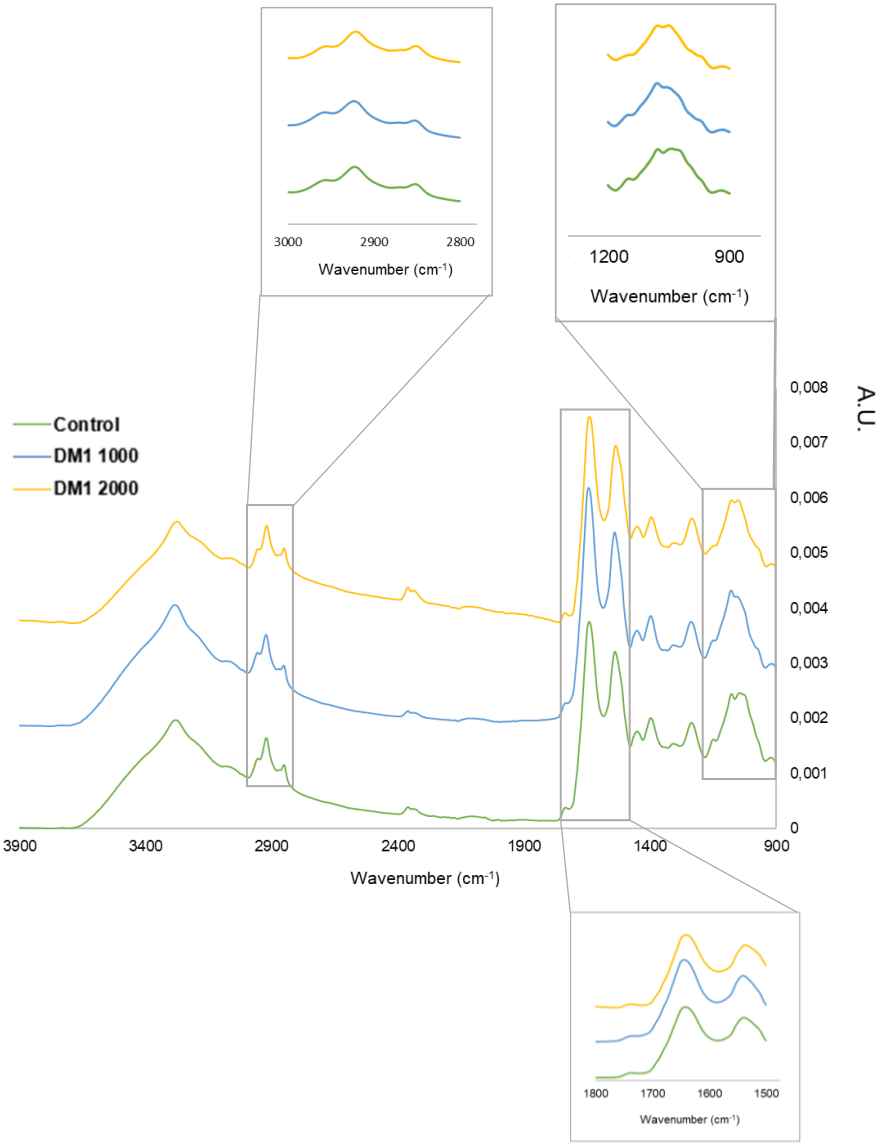


Figure III.1 - **Average FTIR Spectra of human DM1 models**. Baseline-corrected and area normalized spectra of human fibroblasts lysates from a healthy and two DM1 donors. Three technical replicates for control (CTG repeat size between 5 and 27), DM1 1000 (CTG repeat size of approximately 1000) and DM1 2000 (CTG repeat size of approximately 2000) fibroblasts were analyzed. (A.U.=Arbitrary Units).

PCA of the spectral region between 3000-2800 cm^{-1}, characterized by lipids, is illustrated in Figure III.2, where the discrimination between the samples is provided by PC2: DM1 1000 and DM1 2000 fibroblasts are in positive PC2, while control is in negative PC2, showing a clear separation between patients and controls of DM1 models. Further, DM1 1000 and DM1 2000 can also be separate by PC1. In this case, DM1 1000 is in positive PC1 whereas DM1 2000 is in negative PC1. The PC2 is the most appropriate discrimination used to separate patients and controls of DM1 models. In this way, by the loadings plots of this spectral region, DM1 1000 and DM1 2000 fibroblasts, differing from controls, are characterized by specific peaks, which corresponds to spectral assignments. In this case, the main spectral assignments that characterize DM1 1000 and DM1 2000 fibroblasts are 2922, 2874 and 2851 cm^{-1}, identified by CH_2 antisymmetric stretching, CH_3 symmetric stretching and CH_2 antisymmetric stretching, respectively. Furthermore, control fibroblasts are characterized by the peaks at 2956, 2931 and 2868 cm^{-1}, identified by CH_3 antisymmetric stretching, CH_2 antisymmetric stretching and CH_3 symmetric stretching, respectively [247,259].

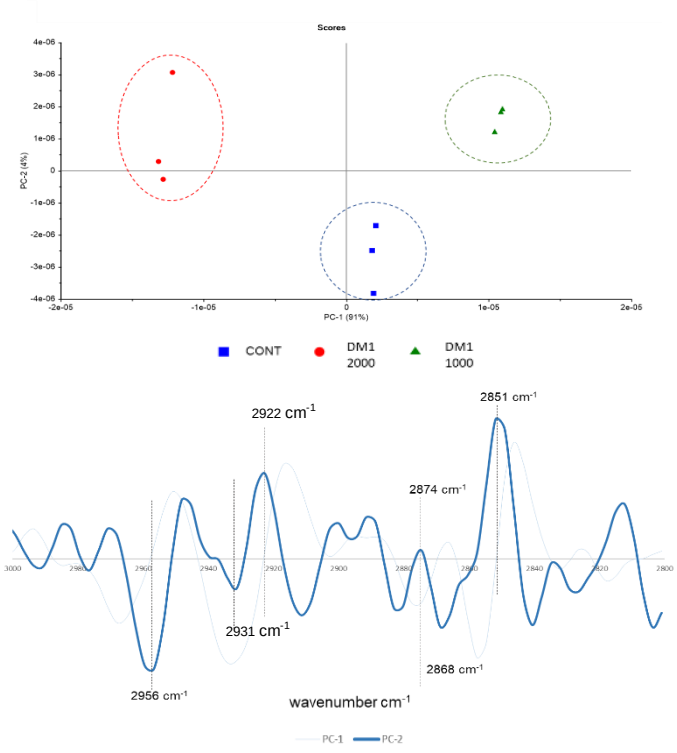


Figure III.2 - PCA scores and loadings plots of ATR-FTIR spectra of human DM1 fibroblasts on the 3000-2800 cm^{-1} region. PCA scores and loadings plots were obtained using the normalized second derivative spectra. Three technical replicates for control (CTG repeat size between 5 and 27), DM1 1000 (CTG repeat size of approximately 1000) and DM1 2000 (CTG repeat size of approximately 2000) fibroblasts were analyzed on the 3000-2800 cm^{-1} region.

PCA of the spectral region between 1800-1500 cm^{-1}, characterized by ester bonds of cholesterol and phospholipids and proteins, is illustrated in Figure III.3, where the discrimination between the samples is provided by PC2: DM1 1000 and DM1 2000 fibroblasts are in positive PC2, while control is in negative PC2. By the loadings plots of this spectral region, DM1 1000 and DM1 2000 fibroblasts, differing from controls, are characterized by specific peaks, which corresponds to spectral assignments. In this case, the main spectral assignments that characterize DM1 1000 and DM1 2000 fibroblasts are 1741, 1656, 1622 and 1546 cm^{-1}, identified by saturated ester C=O stretching (ester functional groups in lipids), α-helix, β-sheets/intermolecular, β-sheets/aggregated strands and amide II, respectively. Furthermore, control fibroblasts are characterized by the peaks at 1682, 1648, 1634 and 1554/1537 cm^{-1}, identified by antiparallel β-sheet/β-turns, α-helix, β-sheet/random coils and amide II, respectively [247,259].

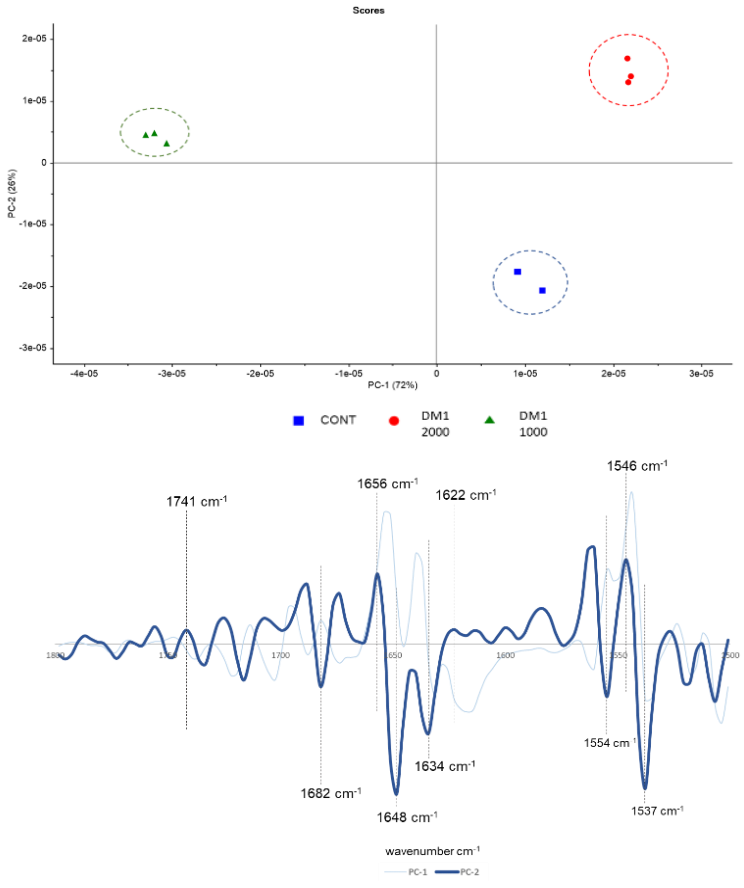


Figure III.3 - **PCA scores and loadings plots of ATR-FTIR spectra of human DM1 fibroblasts on the 1800-1500 cm^{-1} region.** PCA scores and loadings plots were obtained using the normalized second derivative spectra. Three technical replicates for control (CTG repeat size between 5 and 27), DM1 1000 (CTG repeat size of approximately 1000) and DM1 2000 (CTG repeat size of approximately 2000) fibroblasts were analyzed on the 1800-1500 cm^{-1} region.

PCA of the spectral region between 1200-900 cm^{-1} is illustrated in Figure III.4, where the discrimination between the samples is provided by PC1: control and DM1 1000 fibroblasts are in positive PC1, while DM1 2000 fibroblasts are in negative PC1. However, DM1 fibroblasts are close to 0 value, presenting a minimal contribution to the classification model presented. In this way, by the loadings plots of this spectral region, the control and DM1 2000 fibroblasts are differently characterized by specific peaks. In this case, the main spectral assignments that characterize control fibroblasts are 1138, 1090, 1047 and 982 cm^{-1}, identified by C–O stretching, PO_2– ionized symmetric stretching (fully H-bonded) of phosphodiester groups, C–O stretching (coupled with C–O bending of the C–OH groups of carbohydrates) and C–N^+–C stretching (nucleic acids), respectively. Furthermore, DM1 2000 fibroblasts are characterized by the peaks at 1155, 1076 and 1020 cm^{-1}, identified by C–O stretching, PO_2–ionized symmetric stretching (fully H-bonded) of phosphodiester groups and C–O stretching (coupled with C–O bending of the C–OH groups of carbohydrates), respectively [247,259].

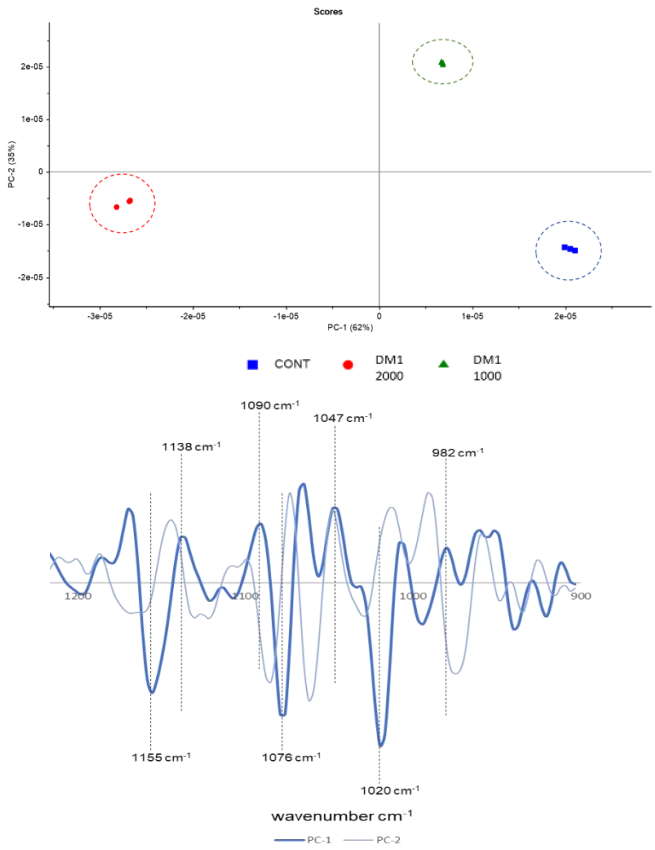


Figure III.4 - PCA scores and loadings plots of ATR-FTIR spectra of human DM1 fibroblasts on the 1200-900 cm^{-1} region. PCA scores and loadings plots were obtained using the normalized second derivative spectra. Three technical replicates for control (CTG repeat size between 5 and 27), DM1 1000 (CTG repeat size of approximately 1000) and DM1 2000 (CTG repeat size of approximately 2000) fibroblasts were analyzed on the 1200-900 cm^{-1} region.

3.4. Discussion

In the work here presented, we performed a FTIR spectroscopy analysis to characterize the biomolecular profile of cultured fibroblasts obtained from DM1 patients. The obtained analyses on the spectral patterns revealed that DM1 fibroblasts present an individual variation at the molecular level. These alterations were mainly observed in the protein and lipids structures. The spectral region between 3000-2800 cm^{-1}, characterized by lipids abortion (Figure III.2) presents differences between the three cell lines used (controls and DM1 donors). In this case, there is a clear discrimination in the identified spectral assignments between both DM1 donors and controls, provided by PC2 analysis. In this way, the identified spectral assignments in the controls can be distinguished from those observed in DM1 1000 and DM1 2000 donors. The main difference identified by PCA scores and loadings plots is in the type of C-H stretching. Indeed, DM1 donors are identified by a higher number of CH_2 stretching, which can be associated to a larger lipidic stretching, while controls are identified by a higher number of CH_3 stretching, which can be associated to a shorter lipidic stretching. Interestingly, mis-splicing events in genes involved in lipid metabolism was found in a mouse model of DM1, including the *LPIN1* gene (encoding lipin protein). Indeed, lipins are phosphatidic acid (PA) phosphatases that catalyze the dephosphorylation of PA, converting PA into diacylglycerol (DAG), an important intermediate that trigger the storage of lipid triacylglycerol (TAG) and the production of the membrane phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [260–262] which may be dysregulated in DM1 donors.

The spectral region between 1800-1500 cm^{-1}, characterized by ester bonds of cholesterol and phospholipids and proteins (Figure III.3) presents differences between the three cell lines used (controls and DM1 donors). In this case, there is a clear discrimination in the identified spectral assignments between both DM1 donors and controls, provided by PC2 analysis. By the PCA scores and loadings plots is possible to observe differences between DM1 donors and controls, mainly on saturated ester C=O stretching. Both DM1 donors can be characterized by saturated ester groups present in lipids, since this type of lipidic stretching no exists in the controls. The obtained results may be explained by alterations on lipid metabolism via lipins, such in the spectral region between 3000-2800 cm^{-1} previous analysed. Moreover, there is an observable difference in the assignments that are related to protein, namely, in the protein conformations that differs between controls and DM1 donors. In both DM1 donors have been identified β-sheets/aggregated strands and β-sheets/intermolecular structures. In particular, the

peak at 1622 cm^{-1} is specifically associated to the presence of protein aggregates [259]. The identified aggregated strands in both DM1 donors can be associated to aggregated complexes described in DM1. Indeed, the CTG expansion with an abnormal increased size is transcribed into RNA but not translated. This mutant RNA accumulates in the nucleus where it forms aggregates, trapping specific RNA binding proteins (CUG-BP1 and MBNL proteins) and leading to the formation of aggregated complexes [33,34]. Also, RAN translation across the described mutant RNA can result in the accumulation of DM1 polyglutamine expansion proteins (polyQn) as toxic nuclear aggregates, demonstrating that aggregated strands could be related both to a RNA toxic gain-of-function and toxicity of RAN proteins [35]. In the other hand, the controls are characterized by the presence of antiparallel β-sheets, structures present in less soluble proteins that may have a tendency to aggregate. Despite the presence of the spectral assignment associated with β-sheets, this could be explained by the fact that most proteins exhibit a mixture of secondary structure.

In the spectral region between 1200-900 cm^{-1} (presented in Figure III.4) can be made discrimination between controls and DM1 donors through PC1 analysis. However, make assignments to the obtained peaks for all these samples has yet not been possible. We cannot infer relevant information about the biochemical changes in controls and DM1 donors, independently of this spectral region being characterized by a large number of different biomolecules, namely fatty acids, nucleic acids, sugars and amino acids [240].

In this study, ATR-FTIR analysis were successfully applied for the first time in the DM1 patient's fibroblasts. The results clearly indicate that there are differences between the controls and the DM1 patient's cell lines at the structural level. Since the biochemical composition of the DM1 cell lines can be distinguished by ATR-FTIR, it could be useful in providing molecular insights on DM1 and also in the development of novel therapeutic strategies. However, more studies should be performed to increase the sample size, and other biological samples should be considered for analysis, such as human urine, serum and plasma, in order to evaluate its potential in the diagnosis of DM1.

CHAPTER IV: Nuclear envelope alterations of DM1 models

4.1. Introduction

DM1 is a multisystemic genetic disorder caused by a CTG repeat expansion in the 3′ untranslated region of the *DMPK* gene, originating mRNA accumulation as nuclear foci [29]. The muscle diseases, including DM1, can be associated to mispositioning of myonuclei since the nuclei remain within the center of the muscle cell, and the myonuclear movement is compromised [36].

Recent studies, have been demonstrated that fibroblasts of DM1 patients presents an increased nuclear size, altered shape of nuclei, and altered localization of the NE proteins emerin, lamin A/C and B1 [73]. Moreover, in DM1 myoblasts patients, no alterations in the localization of some NE proteins including SUN1, SUN2, LBR, nesprin1 and nesprin2 were observed [234]. In this way, further studies are necessary to disclose the contribution of the NE in DM1 given that DM1 mutations might affect NE organization and consequently nuclear morphology. The group of laminopathies diseases, associated to mutations in genes encoding lamin or lamin-binding proteins, crucial components of the NE, have been associated to nuclear abnormalities including nuclear blebbing, resulting in fragile and mechanically unstable nuclei, and also an altered nuclear shape, revealing that NE may play an important role in determining nuclear architecture [73,145,263,264].

Therefore, the main aim of this study is to investigate the nuclei alterations and the localization of NE proteins lamin A/C, emerin, SUN1 and LAP1, in DM1 human fibroblasts, in order to provide new insights on the contribution of NE for DM1 pathophysiology.

4.2. Materials and Methods

4.2.1. Samples

Fibroblasts from biopsies from donors with different CTG repeat sizes: apparently healthy, with a CTG repeat size between 5 and 27 (GM02673), clinically affected with a CTG repeat size of approximately 1000 - DM1 1000 (GM04647) and clinically affected with a CTG repeat size of approximately 2000 - DM1 2000 (GM03989) were obtained from Coriell Institute. All the donors were male and Caucasian. The human patient's fibroblasts included in this study have 1000 and 2000 CTG repeat size, representing the classical adult and congenital DM1 subtypes, respectively.

4.2.2. Cell culture

Fibroblasts were cultured in DMEM (Dulbecco's Modified Eagle Medium, high glucose) (Gibco™) supplemented with 15% FBS (Fetal Bovine Serum, Origin South America) (Gibco™). All the washes performed were made using Dulbecco's 1X PBS (Phosphate Buffered Saline), without Ca^{2+} and Mg^{2+} (BioConcept Ltd. Amimed®). Fibroblasts were sub cultured whenever 85-90% confluence was reached and then incubated 0.05% Trypsin-EDTA (Gibco™) for 5 min to separate the cells from the cell flask whenever subculture was needed. Cell cultures were maintained at 37ºC under 5% CO_2. Fibroblasts were maintained until passage number 14 was reached. At passage number 12 cells were prepared for immunocytochemistry (see below).

4.2.3. Antibodies

The following primary antibodies were used: lamin A/C, emerin, LAP1 and SUN1 (see table IV.1 for further details) for immunocytochemistry (ICC) analysis.

Table IV.1 - **Primary antibodies used in order to detect the multiple proteins.**

Antibody	Antibody company	Antibody type	Target	Dilution
Lamin A/C Antibody (E-1) sc-376248	Santa Cruz Biotechnology	Mouse, monoclonal	Lamin A/C	1:250
Emerin Antibody (H-12) sc-25284	Santa Cruz Biotechnology	Mouse, monoclonal	Emerin	1:500
LAP1 Antibody	Atlas Antibodies	Rabbit, polyclonal	LAP1	1:150
SUN1 Antibody	Kindly provided by Ya - Hui Chi	Rabbit, polyclonal	SUN1	1:500

The secondary antibodies used were Invitrogen Alexa Fluor 488 or 594 dye from Thermo Fisher Scientific (mouse or rabbit, accordingly).

4.2.4. Immunocytochemistry

Fibroblasts at passage 12 (PN12) were grown in 6-well plates (Corning) with coverslips. Then, cells were washed once with 1x PBS and 4% paraformaldehyde (PFA) was added for 20 min in a ventilated hood to each well of the plate (cell fixation). Cells were washed three times with 1x PBS. Then the cells were permeabilized using 0.2% Triton-X in 1x PBS for 10 min. After that, cells were washed with 1x PBS and incubated with 3% BSA in 1x PBS (blocking solution) for 1h, to avoid unspecific binding. Further, the primary antibodies were incubated for 2h at RT. Cells were washed with 1x PBS and the adequate fluorescence secondary antibodies were incubated for 1h in the dark. A final wash was performed with 1x PBS. Coverslips were mounted on a microscope slide with VECTASHIELD® Mounting Media with 4',6-diamidino-2-phenolyde (DAPI) (Vector Laboratories). Image acquisition of coverslips was performed using an epifluorescence microscopy Zeiss AxioImager Z1 (Zeiss) motorized microscope equipped with a Plan-ApoCHROMAT 63x/1.40 oil objective lens. Photographs were taken with a digital AxioCam HR3 (Soft Imaging System).

4.2.4.1. Data Analysis and Statistics

Quantitative analyses of the acquired images were performed using Fiji / ImageJ software (32-bit mode). Initially, the image scale was altered to 6.3 pixels/µm, then each image was converted to 8-bit format, and adjusted the brightness, contrast and specific threshold for the observed nuclei. Automatic quantification of the nuclear area and the nuclear circularity [(4π x area)/perimeter^2] parameters were calculated using the information obtained in the window "Analyze'/'Analyze Particles″. Statistical significance analysis was conducted using GraphPad Prism 6 software and data were analyzed using one-way ANOVA followed by the Dunnett's multi-comparisons test, for comparison of data with control.

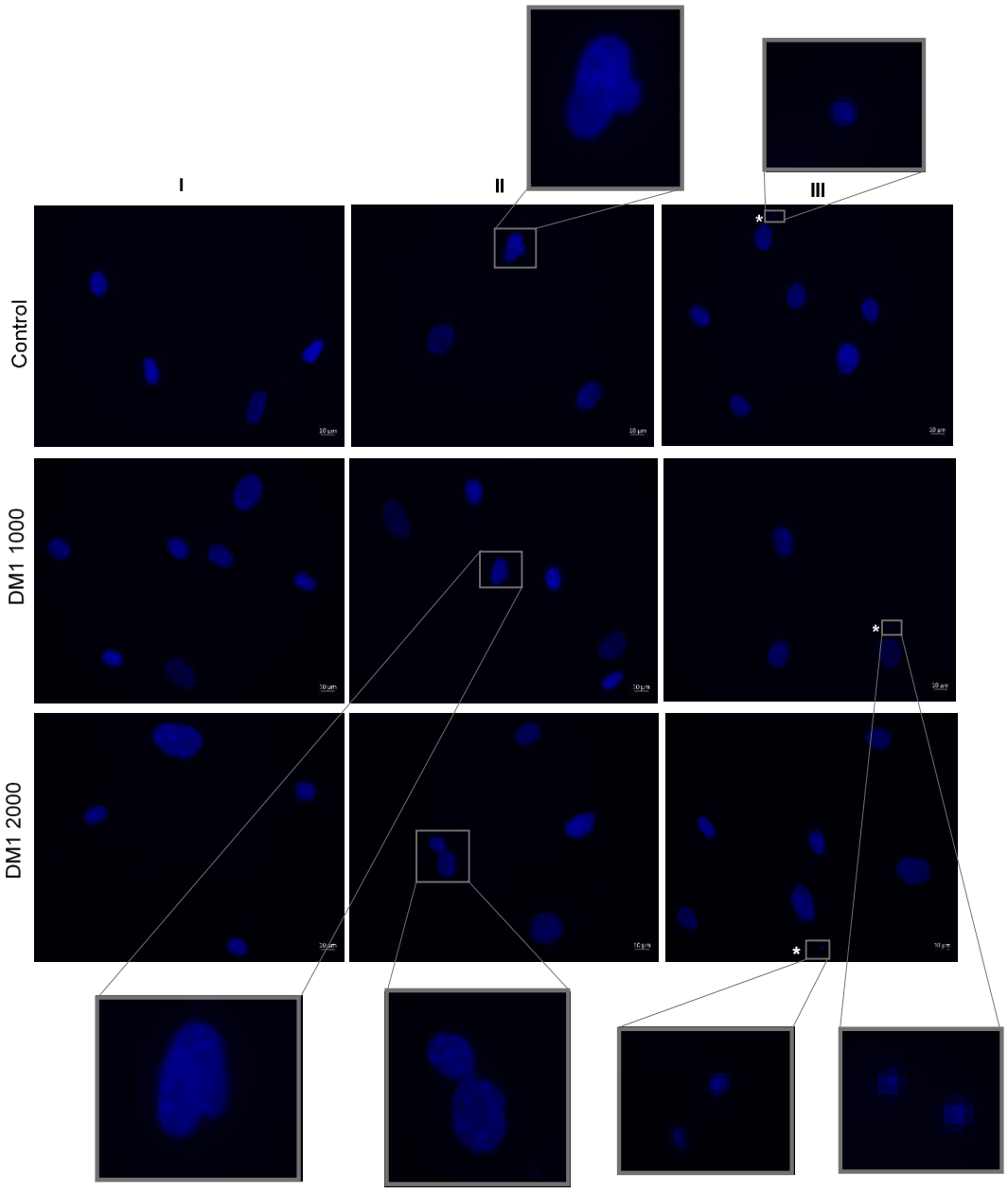
4.3. Results

4.3.1. Evaluation of the nuclear profile in DM1 cellular models

In order to investigate nuclei alterations in DM1 human fibroblasts, immunocytochemistry was performed. Briefly, human fibroblasts from donors with a CTG repeat size between 5 and 27 (healthy), a CTG repeat size of approximately 1000 (DM1 1000) and a CTG repeat size of approximately 2000 (DM1 2000) at passage number 12 were stained with DAPI, and their nucleus analysed. In particular, the nuclear circularity, nuclear area and the presence of deformations and micronucleus were determined (Figure IV.1). Nuclear circularity, also termed nuclear contour ratio, is a quantitative measure of the circular shape of the nucleus. The nuclear counter ratio has a maximum value of 1.0 that corresponds to perfect circle. For the quantification of deformed nuclei it was taken into account the presence of nucleus with blebs and misshaped nuclei. The presence of micronucleus was quantified when additional small nucleus is visible in the cell.

The mean of the nuclear area (represented in Figure IV.1B) presents a slight increase in DM1 1000 fibroblasts, comparing to the control (244,638 μm^2 and 224,373 μm^2, respectively) whereas the DM1 2000 fibroblasts presents a slight decrease comparing to the control (217,136 μm^2 and 224,373 μm^2, respectively). From these results we could realize that the mean of the nuclear area is around 200 μm^2. Therefore, we proceed with the evaluation of quantification of the number of cells <200 μm^2 and >200 μm^2.The mean of the nuclear area of cells with area inferior to 200 μm^2 decreases in both DM1 donors, being significant in the DM1 1000 fibroblasts (p-value 0.0242, mean of nuclear area 159.574 μm^2), comparing to the control. The mean of the nuclear area of cells with area superior to 200 μm^2 increases significantly in both DM1 1000 and DM1 2000 fibroblasts (p-value 0.0032, mean of nuclear area 302.163 μm^2; p-value 0.0328, mean of nuclear area 282.18 μm^2, respectively). Regarding the % of cells with deformed nuclei (represented in Figure IV.1C), is observed a tendency to increase in DM1 1000 and DM1 2000 fibroblasts, comparing to the control. The overall nuclear circularity (represented in Figure IV.1D) was not affected, comparing the control and both DM1 donors. The % of cells with micronuclei formation (represented in Figure IV.1E) increases in both DM1 1000 and DM1 2000 fibroblasts, comparing to the control, although this increase is not significant.

A.



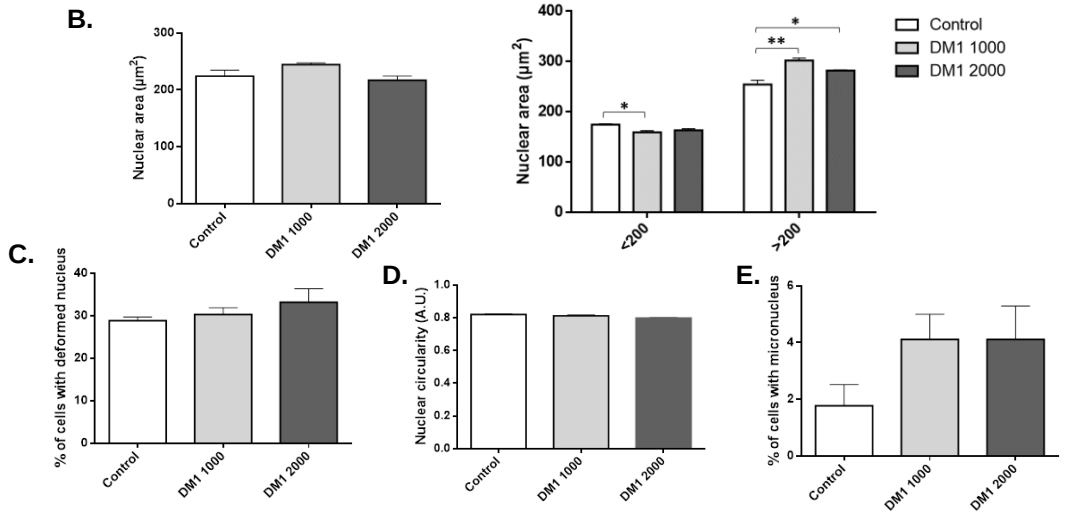
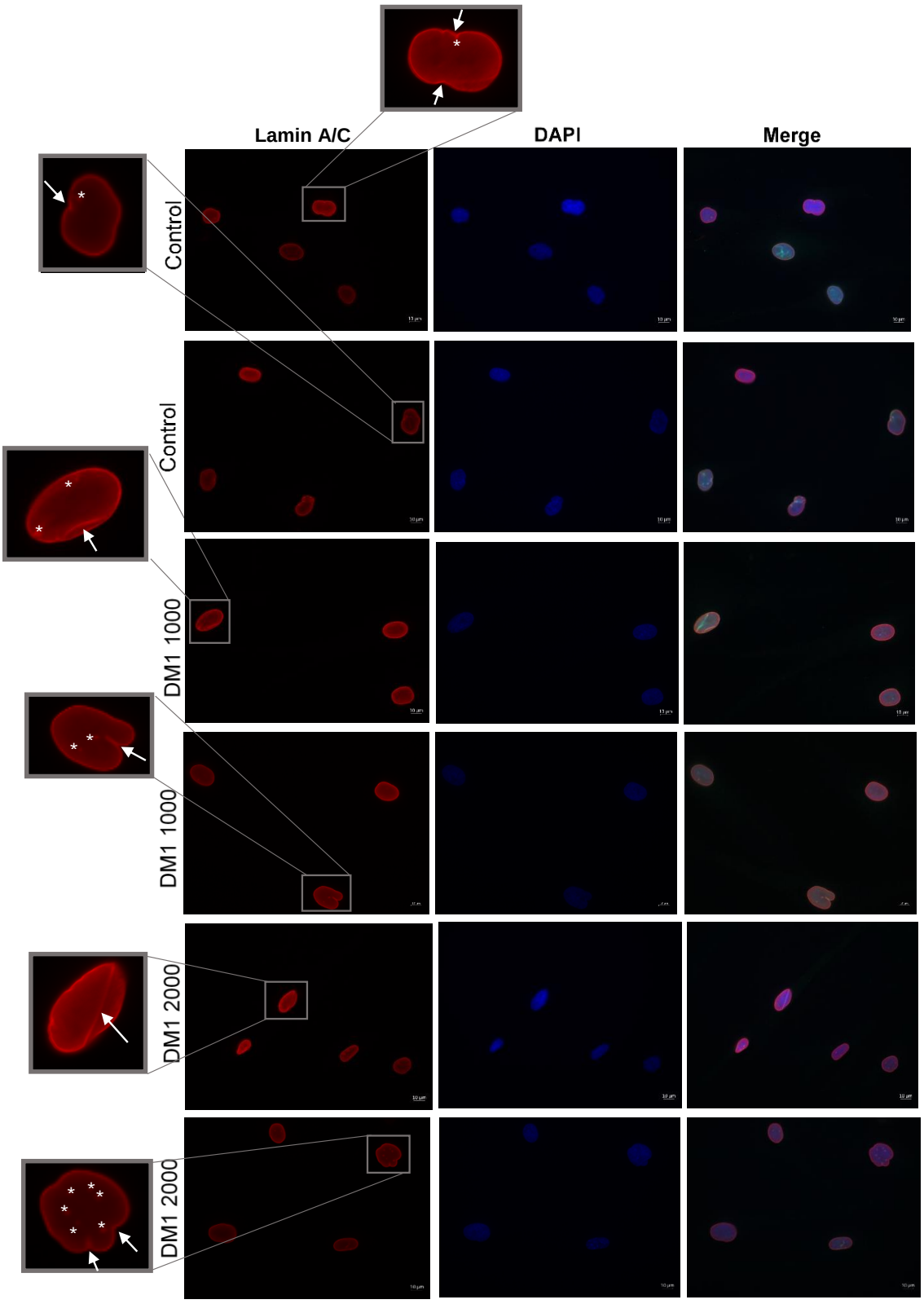


Figure IV.1 - Nuclear profile of DM1 human fibroblasts (A). Nuclear profile of human fibroblasts from a healthy and two DM1 donors were analyzed by microscopy analysis. Nuclei were stained using DAPI (blue). Evaluation of nuclear area **(B)**, deformed nucleus **(C)**, nuclear circularity **(D)** and micronucleus **(E)**. The quantitative data are presented as mean ± SEM and was obtained by analyzing 100 cells per condition from 9 independent experiments. Statistical analysis was performed by comparison between the control (CTG repeat size between 5 and 27), DM1 1000 (CTG repeat size of approximately 1000) and DM1 2000 (CTG repeat size of approximately 2000) fibroblasts by using one-way ANOVA test followed by the Dunnett's test. Statistical significance symbol represents *p < 0.05, ** p < 0.01. **(I)** – Nuclear area representation, **(II)** – Deformed nuclei representation; **(III)** – Micronuclei representation. (A.U.=Arbitrary Units; * - micronucleus). Scale bar, 10µm.

4.3.2. Nuclear envelope proteins localization in DM1 cellular models

In order to investigate the localization of NE proteins in DM1 models, immunocytochemistry was performed. Briefly, human fibroblasts with CTG repeat size between 5 and 27 (control), CTG repeat size of approximately 1000 (DM1 1000) and CTG repeat size of approximately 2000 (DM1 2000), at passage number 12, were labelled with lamin A/C (Figure IV.2), emerin (Figure IV.3), SUN1 (Figure IV.4) and LAP1 (Figure IV.5).

A.


Lamin A/C
DAPI
Merge
Control
Control
DM1 1000
DM1 1000
DM1 2000
DM1 2000

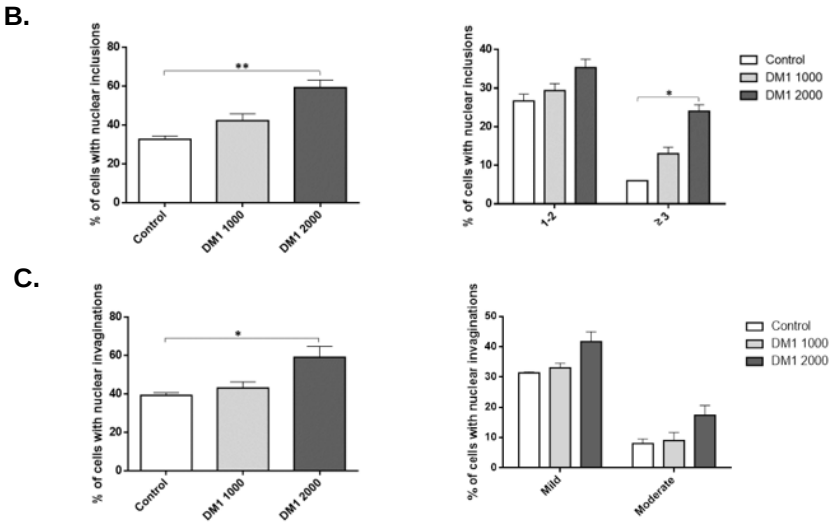
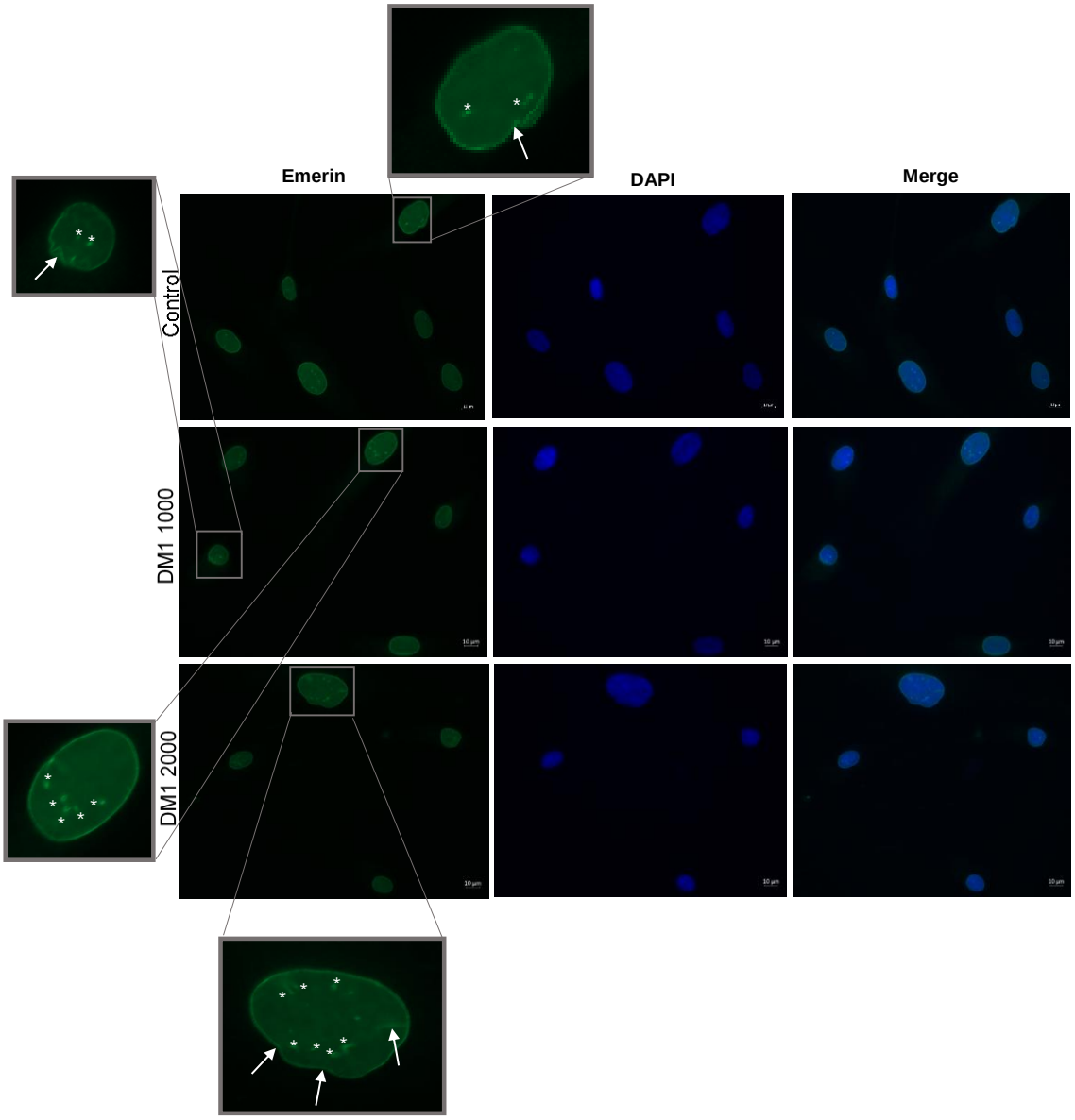


Figure IV.2 - Subcellular distribution of lamin A/C in DM1 human fibroblasts (A). Subcellular distribution of lamin A/C in human fibroblasts from a healthy and two DM1 donors were analyzed by immunocytochemistry. Lamin A/C was detected using an anti-mouse Alexa-594 conjugated secondary antibody (red). Nucleic acids were stained using DAPI (blue). **(B)** Evaluation of nuclear inclusions and nuclear invaginations **(C)**. The quantitative data are presented as mean ± SEM and was obtained by analyzing 100 cells per condition from 3 independent experiments by comparison between the control (CTG repeat size between 5 and 27), DM1 1000 (CTG repeat size of approximately 1000) and DM1 2000 (CTG repeat size of approximately 2000) by using one-way ANOVA test followed by the Dunnett's test. Statistical significance symbol represents *p < 0.05, ** p < 0.01. (Arrow – nuclear invaginations; * - nuclear inclusions). Scale bar, 10µm.

Lamin A/C is a NE protein located in the inner part of the nucleus where is connected to chromatin and INM proteins. Our study reveals an altered localization of lamin A/C in the NE in DM1 1000 and DM1 2000 (Figure IV.2A). The parameters lamin A/C-positive nuclear inclusions and lamin A/C positive-nuclear invaginations are presented in Fig. IV.2B and C, respectively. The % of cells that presents lamin A/C-positive nuclear inclusions increase significantly in DM1 2000 fibroblasts (p-value 0.0029), and DM1 1000 however with letter no significant increase is observed, comparing to the control. Further, we decided to evaluate the number of inclusions establishing two categories (1-2 and ≥3). The % of cells with 1-2 nuclear inclusions tends to increase in both DM1 donors, comparing to the control, although this increase is not significant. The % of cells with 3 or more nuclear inclusions increase in both DM1 donors, comparing to the control, however, a significant increase in DM1 2000 fibroblasts (p-value 0.0178) is noted. These nuclear inclusions seem to be located in regions where invaginations of the nucleus occur, in both DM1 donors. The presence of lamin A/C positive-nuclear invaginations is also observed, and increase in DM1 fibroblasts, being significant in the DM1 2000 fibroblasts (p-value 0.0241). The mild nuclear invaginations correspond to the less severe lamin A/C positive-nuclear invaginations, whereas moderate nuclear invaginations correspond to other ones, enclosing the more severe

states. Regarding the type of nuclear invaginations, there is an increase of both mild and moderate invaginations, in DM1 1000 and DM1 2000 fibroblasts, although this increase is not significant.

A.



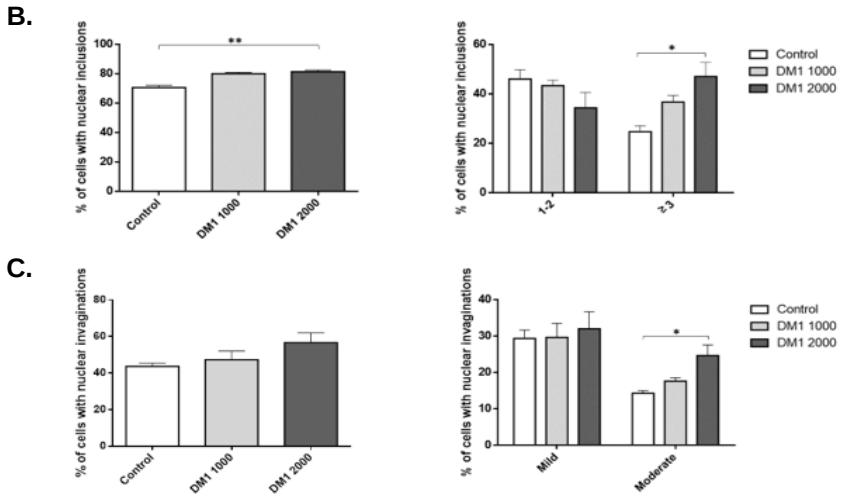
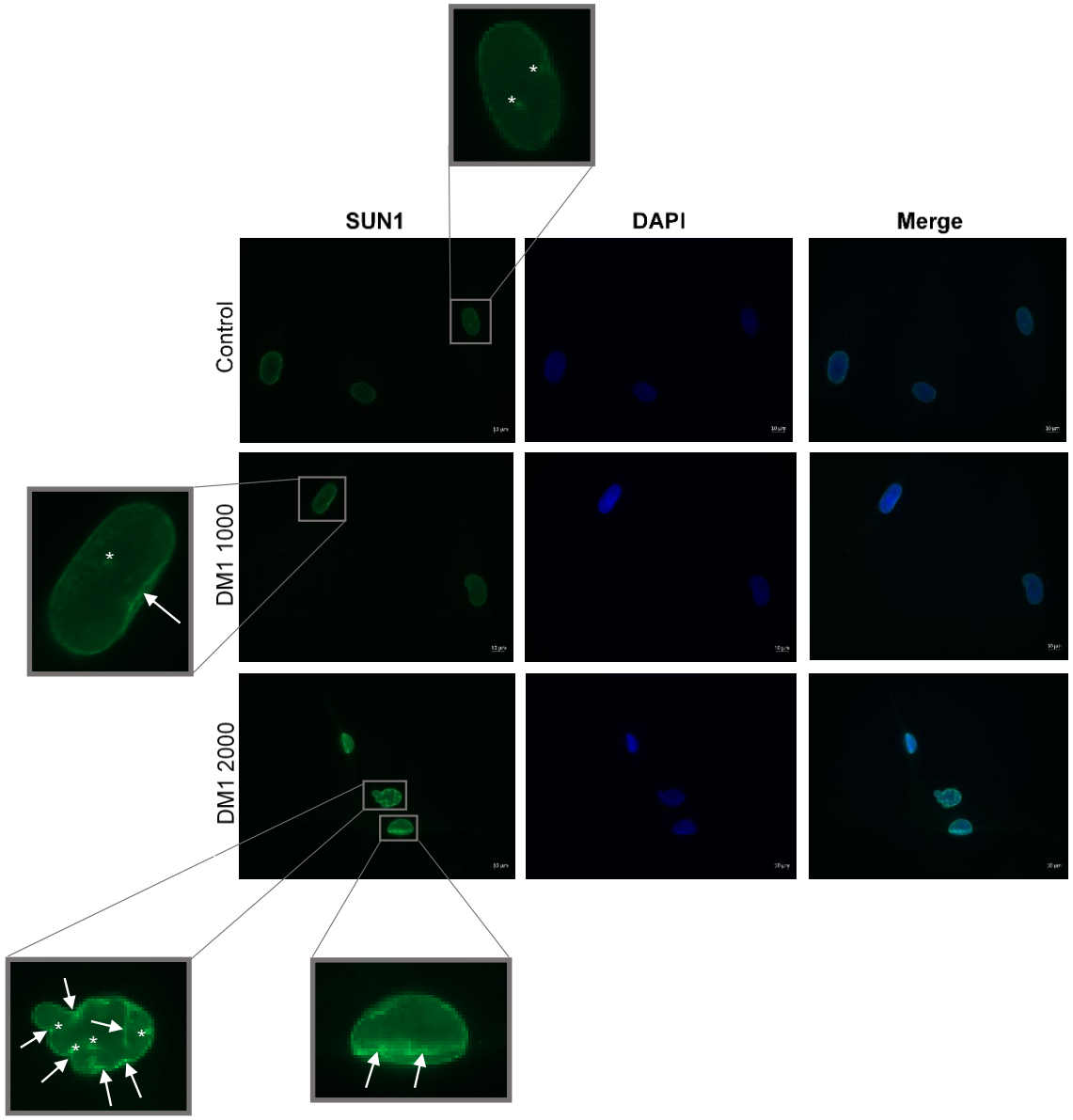


Figure IV.3 - Subcellular distribution of emerin in DM1 human fibroblasts (A). Subcellular distribution of emerin in human fibroblasts from a healthy and two DM1 donors were analyzed by immunocytochemistry. Emerin was detected using an anti-mouse Alexa-488 conjugated secondary antibody (green). Nucleic acids were stained using DAPI (blue). **(B)** Evaluation of nuclear inclusions and nuclear invaginations **(C)**. The quantitative data are presented as mean ± SEM and was obtained by analyzing 100 cells per condition from 3 independent experiments by comparison between the control (CTG repeat size between 5 and 27), DM1 1000 (CTG repeat size of approximately 1000) and DM1 2000 (CTG repeat size of approximately 2000) by using one-way ANOVA test followed by the Dunnett's test. Statistical significance symbol represents *p < 0.05, ** p < 0.01. (Arrow – nuclear invaginations; * - nuclear inclusions). Scale bar, 10µm.

Emerin is an integral NE protein located at the inner membrane and similarly to lamin A/C, an altered localization of emerin is noted in the NE, in DM1 1000 and DM1 2000 (Figure IV.3A). The same parameters previously analysed for lamin A/C were also analysed for emerin, namely emerin-positive nuclear inclusions (Figure IV.3B) and emerin positive-nuclear invaginations (Figure IV.3C), regarding the presence, number (1-2 and ≥3) and type (mild and moderate). In the % of cells with nuclear inclusions, in both DM1 donors there is a tendency to increase, however only in DM1 2000 fibroblasts is significant (p-value 0.0017). Regarding the number of nuclear inclusions, in DM1 fibroblasts the % of cells with 1-2 nuclear inclusions decrease, whereas the % of cells with 3 or more nuclear inclusions increase, being significant in DM1 2000 fibroblasts when compared to control (p-value 0.0200). Is noted a high number of nuclear inclusions in both DM1 fibroblasts when the nucleus presents an irregular shape. The % of cells with nuclear invaginations also increase in DM1 1000 and DM1 2000 fibroblasts, comparing to the control, without statistical significance. Both the mild and moderate type of nuclear invaginations increase in DM1 1000 and DM1 2000 fibroblasts. The increase of moderate nuclear invaginations in DM1 2000 fibroblasts is significant (p-value 0.0185).

A.



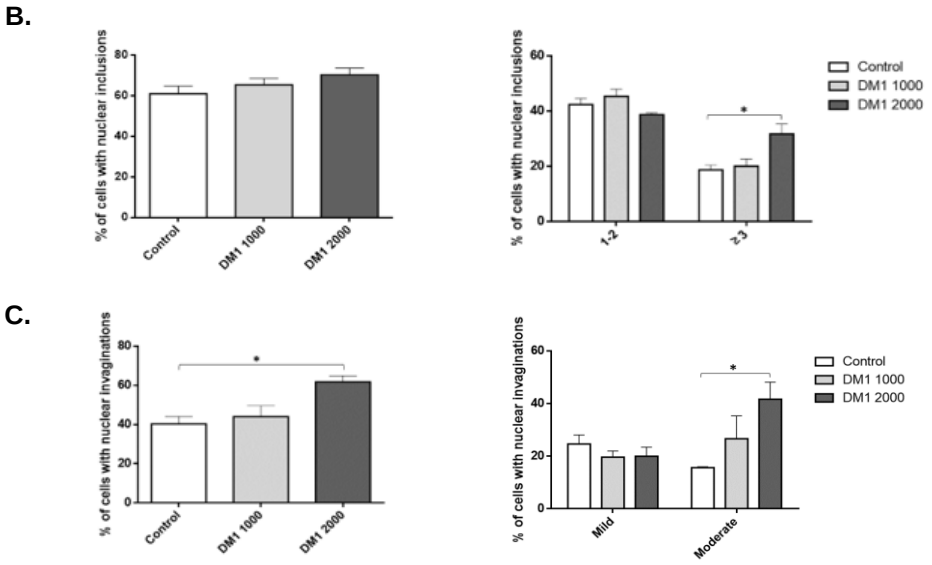
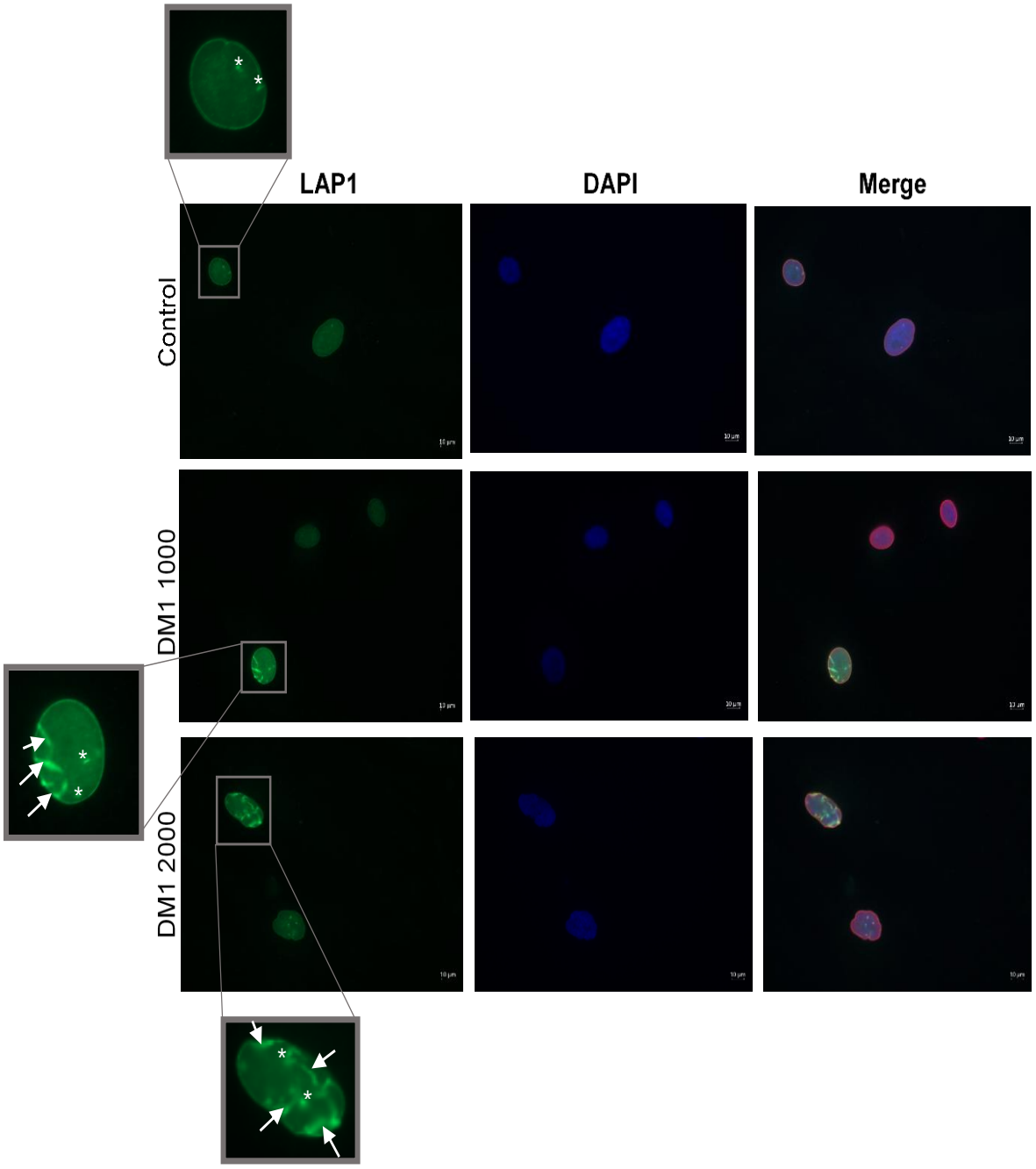


Figure IV.4 – Subcellular distribution of SUN1 in DM1 human fibroblasts (A). Subcellular distribution of SUN1 in human fibroblasts from a healthy and two DM1 donors were analyzed by immunocytochemistry. SUN1 was detected using an anti-rabbit Alexa-488 conjugated secondary antibody (green). Nucleic acids were stained using DAPI (blue). **(B)** Evaluation of nuclear inclusions and nuclear invaginations **(C)**. The quantitative data are presented as mean ± SEM and was obtained by analyzing 100 cells per condition from 3 independent experiments by comparison between the control (CTG repeat size between 5 and 27), DM1 1000 (CTG repeat size of approximately 1000) and DM1 2000 (CTG repeat size of approximately 2000) by using one-way ANOVA test followed by the Dunnett's test. Statistical significance symbol represents *p < 0.05. (Arrow – nuclear invaginations; * - nuclear inclusions). Scale bar, 10µm.

SUN1 is an integral NE protein, and similarly to emerin and lamin A/C, is located at the inner membrane. An altered localization of SUN1 in the NE have been noted in DM1 2000 fibroblasts (Figure IV.4A). The presence of nuclear inclusions and nuclear invaginations tends to increase in DM1 1000 fibroblasts and DM1 2000 fibroblasts, comparing to control. Regarding the nuclear invaginations, this increase is significant in DM1 2000 fibroblasts (p-value 0.0265). In the % of cells with 1-2 nuclear inclusions, there is an increase in DM1 1000 fibroblasts and decrease in DM1 2000 fibroblasts, without statistical significance, comparing to the control. Also, the % of cells with 3 or more nuclear inclusions, tends to increase in both DM1 donors, but only significantly in DM1 2000 fibroblasts (p-value 0.0314), comparing to the control. The nuclear inclusions seem to be located in regions where invaginations of the nucleus occur, evidenced in DM1 2000 fibroblasts. In both DM1 1000 and DM1 2000 fibroblasts, the % of cells with mild nuclear invaginations tends to decrease comparing to control and the % of cells with moderate nuclear invaginations increase in DM1 1000 and DM1 2000 fibroblasts, with statistical significance in the last one (p-value 0.0161).

A.



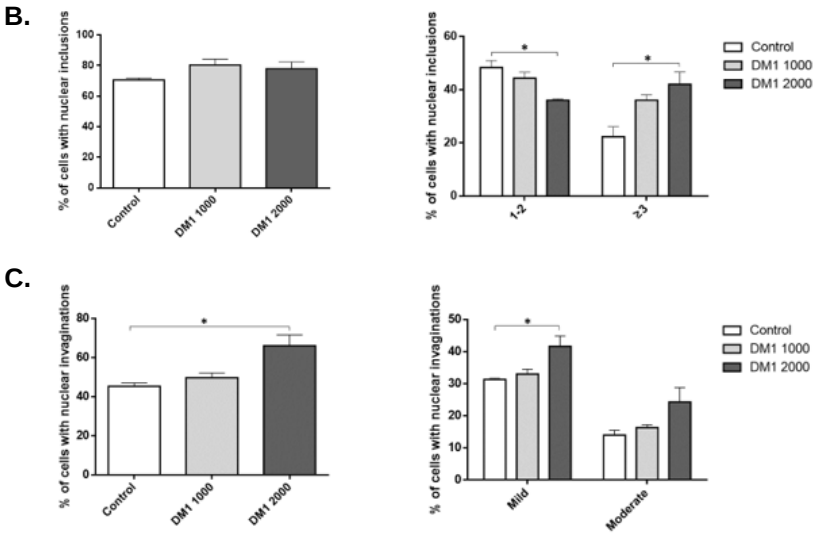


Figure IV.5 – Subcellular distribution of LAP1 in human DM1 fibroblasts (A). Subcellular distribution of LAP1 in human fibroblasts from a healthy and two DM1 donors were analyzed by immunocytochemistry. LAP1 was detected using an anti-rabbit Alexa-488 conjugated secondary antibody (green). Nucleic acids were stained using DAPI (blue). **(B)** Evaluation of nuclear inclusions and nuclear invaginations **(C)**. The quantitative data are presented as mean ± SEM and was obtained by analyzing 100 cells per condition from 3 independent experiments by comparison between the control (CTG repeat size between 5 and 27), DM1 1000 (CTG repeat size of approximately 1000) and DM1 2000 (CTG repeat size of approximately 2000) by using one-way ANOVA test followed by the Dunnett's test. Statistical significance symbol represents *p < 0.05. (Arrow – nuclear invaginations; * - nuclear inclusions). Scale bar, 10µm.

LAP1 is another integral NE protein located at the inner surface. This protein shows an altered localization in the NE in both DM1 fibroblasts (DM1 1000 and DM1 200) (Figure IV.5A). There is a tendency to increase in the presence of nuclear inclusions in both DM1 1000 fibroblasts and DM1 2000 fibroblasts, comparing to the control, although without statistical significance. Regarding the % of cells with only 1-2 nuclear inclusions, there is a decrease in both DM1 donors but only significantly in DM1 2000 fibroblasts (p-value 0.0123), whereas the % of cells with 3 or more nuclear inclusions increase in both DM1 donors but only significantly in DM1 2000 fibroblasts (p-value 0.0223). Further, is observed an increase in the presence of nuclear invaginations in DM1 1000 and DM1 2000 fibroblasts, with statistical significance only in DM1 2000 fibroblasts (p-value 0.0156). In particular, an increase in the % of cells with mild and moderate nuclear invaginations in DM1 1000 and DM1 2000 fibroblasts is observed, comparing to the control. However, is only significant in mild nuclear invaginations in DM1 2000 (p-value 0.0271).

4.4. Discussion

NE proteins are considered key components of nuclear structure and positioning, playing an essential role in muscle function [36–39]. DM1 is a multisystemic disease characterized mainly by muscle impairments [3,13] and has been associated to NE proteins alterations [73,234,265].

Recent studies propose NE as a novel feature of DM1 given that DM1 patients present an impaired nuclear structure and mislocalization of NE proteins, namely emerin, lamin A/C and lamin B1 [73]. Additionally, NE proteins, including lamin A/C, emerin, LAP1 and SUN proteins have been linked to muscle diseases since are significant components in myonuclear movement regulation and consequently muscle function [36,72]. NE may play an important role in determining nuclear architecture since mutations in genes encoding lamin or lamin-binding proteins, which are essential components in NE integrity, have been related to nuclear abnormalities such as blebs and misshaped nuclei [73,145,263,264].

In the work here presented, we started by evaluating the nuclear profile in DM1 cellular models. Our results evidenced a tendency to increase the number of cells with micronuclei in DM1 donors (Figure IV.1E). Micronuclei can be associated to defects in NE reassembly after its breakdown, a mechanism necessary to eukaryotic cells enter in mitosis. In this way, abnormal incorporation of chromosomes into the main nucleus when the NE is reassembled resulting in their encapsulation into a separate and smaller nucleus designated as micronucleus [87]. These defects in NE reassembly have already been described for the depletion of BAF [87,266], a chromatin-binding protein that interacts with lamin A/C, LAP2, emerin and MAN1 [74,88]. Consequently, it is predicted that defects of these proteins can be associated to an abnormal NE reassembly and consequently micronuclei formation. Another nuclear parameter, the nuclear area was also evaluated in DM1 cellular models and the results are very interesting given that the mean of the nuclear areas seems to be identical in both control and DM1 models, and a significant increase is observed in DM1 models when we analyzed the nucleus with mean of the nuclear area > 200 μm^2 (Figure IV.1B). This result might indicate that the cell cycle is somehow arrested in DM1 models. Microtubules can lead to NE disassembly in early mitosis and nuclear lamins are phosphorylated upon entry into prophase, leading to its breakdown. Therefore, cell division can be associated to a proper microtubules action [267]. Microtubules are a constituent of the cytoskeleton, essential for proper nuclear positioning that is dependent on NE proteins [267]. Since muscle diseases can be characterized by mispositioning of nuclei [36], it is predicted that a defective

microtubules action and consequently altered cell division, which may explain the increase of the nuclear area in DM1 fibroblasts (DM1 1000 and 2000). The nuclear circularity (Figure IV.1D) and the presence of cells with nuclear abnormalities or deformed nuclei (misshape nucleus or blebs; Figure IV.1C) were also evaluated. The results indicated that nuclear circularity has identical values in the three cellular models studied, demonstrating that is a parameter not affected by the disease. The number of cells with nuclear abnormalities or deformed nuclei increase in DM1 donors, demonstrating abnormal nuclei as a relevant feature to be in account in DM1. Additionally, these results are similar to those observed in a muscular dystrophy, given that fibroblasts derived from Emery-Dreifuss muscular dystrophy (EDMD) patients often have an abnormal nuclear shape and the presence of blebs [268,269]. In this way, myotonic dystrophies may also be associated with a deformed nuclear structure, evidenced by the results here obtained. Nuclear blebs, characteristic of a deformed nucleus, are protrusions from the normal circular/ellipsoidal nucleus which are connected with lamin and chromatin alterations and involved in diseases that present anomalous nuclear morphology such as laminopathies [270]. Indeed, the presence of nuclear blebs have already been observed in lamin mutations and depletion [150,271–273], demonstrating the important role that the nuclear lamina, an essential component of the nuclear envelope, may play in the development of these abnormal structures. Since our study showed an altered localization of lamin A/C in DM1 donors (Figure IV.2.A), alterations in this protein may be associated with the formation of blebs and consequently deformed nuclei.

In the work here presented, the localization of some NE proteins, including lamin A/C, emerin, SUN1 and LAP1, was also evaluated in DM1 cellular models. For all these proteins is noted an altered localization in the NE of DM1 fibroblasts. In general, the number of protein positive-nuclear inclusions and protein positive-nuclear invaginations seems to be related to abnormal nuclear shape and consequently a high number of these nuclear inclusions and invaginations are associated with more deformed nuclei observed in DM1 models (Figures IV.2-5). According to our study, lamin A/C showed an altered localization in NE (Figure IV.2A), in both DM1 fibroblasts (DM1 1000 and DM1 200), which is in agreement with previous studies [73]. The assessment of lamin A/C positive-nuclear inclusions and lamin A/C positive-nuclear invaginations was also evaluated. In fact, this protein shows a very significant increase in the presence of lamin A/C positive-nuclear inclusions, visible in DM1 2000 compared to the control. Although the % of cells with lamin A/C positive-nuclear invaginations also showed a significant increase in DM1 2000 compared to the control, it is apparent that the % of cells with mild nuclear

invaginations is much higher in the three DM1 cellular models, when comparing to moderate nuclear invaginations, which present lower values, highlighting that lamin A/C positive-nuclear inclusions are more evident in the altered localization of this protein in NE.

Furthermore, our studies also demonstrated an altered localization of emerin in NE (Figure IV.3A) in DM1 donors, which is in line with previous studies [73] and is noted an accumulation in emerin positive-nuclear inclusions, particularly in the DM1 1000 and DM1 2000 fibroblasts. In fact, in the % of cells with emerin positive-nuclear inclusions, there is a tendency to increase in both DM1 fibroblasts comparing to the control with a high significant increase in DM1 2000 fibroblasts. In addition, the % of cells with ≥3 inclusions in DM1 2000 have a significant increase comparing to the control and it is notorious that the % of cells with mild nuclear invaginations is higher in the three DM1 cellular models, when comparing to moderate nuclear invaginations. Lamin A/C interacts with emerin at the INM, regulating chromatin organization via their common interactor - Barrier to Autointegration Factor (BAF) [274]. Indeed, chromatin attachments to the lamina have been mapped at high resolution in *D. melanogaster*, mouse and human cells [275]. Chromatin consists of DNA, RNAs that contribute to its structure and regulation in the cell nucleus and a large number of proteins such as histones. Maintenance of chromatin structure and its proper regulation is an essential cellular requirement. However, this structure can be altered if cells undergo an irreversible proliferation arrest which can be triggered by DNA damage, resulting in senescence and alterations in chromatin structure. Senescent cells form foci - senescence associated heterochromatin foci (SAHF) - which have diverse physiological functions: tumor suppression, proliferation arrest, and suppressing of DNA damage signaling to prevent cell death [275,276]. In this way, accordingly to our previous results, DM1 donors shows a tendency to have an increase area, which might indicate that the cell cycle is arrested. Since DM1 is triggered by an abnormal increase of CTG repeats, is predicted that this DNA damage is implicated in this process. Besides, given that lamin A/C and emerin show an altered localization in NE of DM1 donors, is possible a correlation between its alteration and dysregulation of chromatin which may be involved in the formation of nuclear inclusions (senescence associated heterochromatin foci - SAHF) and consequently we might speculate that DM1 is associated with cellular senescence. Indeed, lamin A/C positive-nuclear inclusions and emerin positive-nuclear inclusions presenting a tendency to increase in DM1 donors comparing to the control.

Concerning SUN1, though previous studies have shown that the localization of this protein in NE does not alter between controls and DM1 fibroblasts, our study reveal

an altered localization of this protein (Figure IV.4A) in DM1 2000 fibroblasts. Previous studies have indicated that DM1 patient's CTG repeats has a maximum value of 1500, so is possible induce that the altered localization of SUN1 can only be seen with a higher number of CTG repeats [234]. Based on performed quantifications, SUN1 seems to accumulate in SUN1 positive-nuclear invaginations. In fact, the % of cells with SUN1 positive-nuclear invaginations have a significant increase in DM1 2000 fibroblasts comparing to the control. In addition, there is also a significant increase in the % of cells with moderate nuclear invaginations in DM1 2000 fibroblasts comparing to the control. Interestingly, in the % of cells with SUN1 positive-nuclear inclusions, no changes are observed in the three DM1 cellular models, meaning that this protein is less present in these inclusions. As previous observed in lamin A/C and emerin, DM1 fibroblasts tend to have more deformed nuclei associated with a presence of nuclear inclusions compared to controls, therefore, the same should be expected with SUN1. However, the % of cells with SUN1 positive-nuclear inclusions remain constant in control and DM1 models used. In this way, is expected that this protein may not stain all inclusions that may exist in the nucleus.

Concerning LAP1, our study showed an altered localization of this protein in NE (Figure IV.5A), visible in DM1 1000 and DM1 2000 fibroblasts, which has yet not been described in previous data. As in SUN1, by performed quantifications of protein positive-nuclear inclusions and protein positive-nuclear invaginations, is noted an accumulation in LAP1 positive-nuclear invaginations. In fact, the presence of LAP1 positive-nuclear invaginations has a significant increase in DM1 2000 fibroblasts comparing to the control and the % of cells with moderate nuclear invaginations also tend to increase in DM1 fibroblasts. In addition, no changes have been denoted between the three DM1 cellular models in the % of cells regarding the presence of nuclear inclusions, which, like to SUN1, is possible infer that this protein may not stain all inclusions that can be present in the nucleus.

SUN1 have been recognized as an essential protein to a proper movement mechanism triggered by the LINC complex, given that provides a connection to nuclear structures while acting as a tether for KASH proteins. The coupling of the cytoskeleton to the nucleoskeleton is required for the nuclear positioning and also organization of the cytoskeleton, which is supported by SUN and KASH domain proteins interaction in LINC complexes spanning the NE [277]. Additionally, LAP1 and torsinA have been recently identified as mediators of the assembly of nucleoskeleton and cytoskeleton in LINC complexes, given that LAP1 was identified as torsinA-interacting protein in NE [278,279]. In this way, have been suggested that torsinA might modulate the interactions between

KASH and SUN proteins based on diverse results: torsinA directly interacts with the KASH domains of several nesprins [278,280], is required for the localization of nesprin-3a to the NE [278,280] and SUN1 is involved in the proper localization of torsinA to the NE [278,281]. Moreover, mutations in the LINC Complex components, namely SUN1, SUN2, nesprin1 and nesprin2, have been related to Emery-Dreifuss muscular dystrophy (EDMD) pathophysiology [213,282,283]. Based on our results is predicted that the altered localization seen in SUN1 and LAP1 in NE of DM1 donors can be associated to an abnormal nuclear positioning and nuclear deformations upon mechanical stress since these proteins are key components in LINC complexes and this mechanical stress can lead to a weakened NE. In this way, makes sense that SUN1 and LAP1 accumulate in protein positive-nuclear invaginations.

The work here presented demonstrates that altered NE organization and abnormal nuclear morphology can be directly associated to DM1 pathophysiology. Therefore, further investigation is required for a proper understanding of the role of NE in DM1.

CHAPTER V: General discussion and concluding remarks

Muscle diseases, including DM1, can be associated to an impaired muscle function with mispositioning of myonuclei associated to NE proteins defects since these proteins have been pointed as crucial components controlling the nuclear positioning, nuclear movements and muscle functions [36]. In this way, in the present study it was evaluated the nuclear profile of DM1 human models (nuclear circularity, nuclear area and presence of deformations and micronucleus) and the subcellular localization of NE proteins (lamin A/C, emerin, SUN1, and LAP1) in DM1 human models. The obtained results evidenced a tendency to increase the number of cells with micronuclei and the nuclear area in DM1 donors, comparing to the control. However, nuclear circularity is similar between the control and DM1 models studied. The number of deformed nuclei, associated to blebs and nuclear invaginations, increase in DM1 1000 fibroblasts and DM1 2000 fibroblasts and the formation of blebs is possibly due to nuclear lamina disruption [73,284]. Our study reveals an altered localization of lamin A/C, emerin, SUN1, and LAP1 proteins in NE. Regarding lamin A/C, emerin and LAP1, these proteins showed an altered localization in NE, in both DM1 fibroblasts, which are in agreement to previous studies [73,285] except for LAP1, given that no studies have yet been performed regarding its location in NE. Concerning SUN1, our study shows an altered localization in NE in DM1 2000 fibroblasts. However, previous studies demonstrated no alterations regarding this protein localization in NE of DM1 models [230]. Both lamin A/C and emerin accumulate in protein positive-nuclear inclusions. Indeed, have been show that lamin A/C and emerin regulate chromatin organization via BAF [274]. In DM1 donors, the DNA damage caused by an increase of CTG repeats may trigger a cell cycle arrest which can lead to alterations in chromatin structure and consequently formation of senescent cells related to senescence associated heterochromatin foci (SAHF). In this way, makes sense that lamin A/C positive-nuclear inclusions and emerin positive-nuclear inclusions have an increase in DM1 donors comparing to the control. Our study also reveals an altered localization of LAP1 and SUN1 in NE of DM1 donors, which can be associated to an abnormal nuclear positioning and nuclear deformations upon mechanical stress, given that LAP1 and SUN1 are important proteins involved in a proper movement mechanism triggered by the LINC complex. In this way, makes sense that the SUN1 and LAP1 accumulate in protein positive-nuclear invaginations.

Furthermore, in order to characterize the biomolecular profile of cultured fibroblasts obtained from DM1 patients, was performed a FTIR spectroscopy analysis revealed that DM1 fibroblasts present an individual variation at the molecular level, mainly in protein and lipids structures. Indeed, DM1 donors can be distinguished from controls by a larger lipidic stretching (higher number of CH_2 stretching) and saturated

ester groups (saturated ester C=O stretching) present in lipids, in the 3000-2800 cm^{-1} and 1800-1500 cm^{-1} spectral regions, respectively. A possible explanation can be based on alterations on lipid metabolism. Lipins are phosphatidic acid (PA) phosphatases which are involved in lipid metabolism. In fact, PA phosphatases catalyze the dephosphorylation of PA, converting PA into diacylglycerol (DAG), an important intermediate that trigger the storage of lipid triacylglycerol (TAG) and the production of the membrane phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [260–262]. Remarkably, mis-splicing events in genes involved in lipid metabolism was found in a mouse model of DM1 [73] and NE, as a dynamic structure undergoing processes of growth, breakdown and reassembly during the cell cycle can be directly associated to lipins action [286]. In fact, previous studies show that Smp2, the yeast homologue of mammalian lipin, is a key regulator of nuclear membrane growth during the cell cycle given that loss of *SMP2* gene causes transcriptional upregulation of enzymes involved in phospholipid biosynthesis and consequent expansion of the nucleus [287]. On the other hand, studies based in Lpin-1, showed no effects on NE reassembly and growth, however this protein is required for NE breakdown, a process dependent of lamins disassembly triggered by DAG-dependent PKCs phosphorylation [286,288]. In fact, downregulation of *LPIN-1* by RNAi can lead to dysregulation of lipid synthesis and consequent defects in NE breakdown, abnormal nuclear shape and disorganization of the peripheral ER [286]. In this way, these results may suggest that dysregulation of lipid metabolism can have a role in NE dynamics since abnormal nuclear shape and augmented nuclear area has been denoted in DM1 fibroblasts comparing to the control. Clearly, the demonstration that the occurrence of mis-splicing events in genes involved in lipid metabolism in DM1 fibroblasts and its potential connection with nuclear membrane morphology requires further investigation.

Also, DM1 donors can be associated to the presence of protein aggregates identified as β-sheets/aggregated strands and β sheets/intermolecular structures in the 1800-1500 cm^{-1} spectral region. The identified aggregated strands in both DM1 donors can be associated to aggregated complexes described in DM1. In this disease, CTG repeats with an abnormal increased size is transcribed into RNA but not translated and this mutant RNA accumulates in the nucleus where it forms aggregates, trapping specific RNA binding proteins (CUG-BP1 and MBNL proteins) and consequent formation of aggregated complexes [33,34]. Further, RAN translation across the described mutant RNA can result in the accumulation of DM1 polyglutamine expansion proteins (polyQn) as toxic nuclear aggregates, demonstrating that aggregated strands could be associated both to a RNA toxic gain-of-function and toxicity of RAN proteins [35]. Therefore, these

results clearly indicated that there are differences between the controls and the DM1 patient's cell lines at the structural level, showing that ATR-FTIR analysis were successfully applied for the first time in the DM1 patient's fibroblasts and could be useful in providing molecular insights on DM1, in order to evaluate its potential in the diagnosis of the disease.

To sum up, our study reveals that there are structural differences between the DM1 cellular models used and NE protein alterations and abnormal nuclear morphology directly association with DM1. Therefore, further studies should be performed in order to uncover the role of NE proteins in this disease and better understand the specificity of structural differences identified in DM1 models. In this way, as complementing of this study, the proposed perspectives are:

- Increase the number of DM1 human fibroblasts in order to ameliorate the obtained results regarding ATR-FTIR and also NE protein alterations in DM1 models;

- Evaluate and quantify the levels of the NE proteins between controls and DM1 donors by immunocytochemistry and immunoblotting.

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