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Genotypic and phenotypic characterization of Becker patients with deletion of exons 45 to 55

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Sorbonne Université

École Doctorale Complexité du Vivant

Centre de Recherche en Myologie – U974

Doctoral thesis in Biology

**Genotype and phenotype characterization
of Becker muscular dystrophy patients
with exons 45 to 55 deletion**

Presented by Elena GARGAUN

Defended on 23rd of November 2020

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ABBREVIATIONS

2'OMe	2'O-Méthyle	dystromiR	deregulated micro-RNA in dystrophinopathies
2'OMePS	2'O-méthyle phosphorothioate	eARN	<i>enhancer</i> ARN
AAV	Adeno Associated Virus	eIF3/4A/E/G	<i>eucaryotic Initiation Factor 3/4A/E/G</i>
ABD	<i>Actin binding domain</i>	eNOS	endothelial Nitrique oxide synthase
ADP	Adenosin Diphosphate	eRF	eucaryotic Releasing Factor
Ago	Argonaut	FAD	Flavin adenine dinucleotide
AMPK	<i>AMP-activated protein kinase</i>	FMN	Food and drug administration
AON	Antisense oligonucleotide	GMPc	Flavin mononucleotide Guanosine Monophosphate cyclique
ARN	Ribonucleic Acid	GRMD	<i>Golden Retriever Muscular Dystrophy</i>
ARNg	RNA guide	GTP	Guanosine Triphosphate
ARNm	Ribonucleic Acid messenger	H1/2/3/4	prolines rich region
ATP	Adenosine Triphosphate	HDAC	Histone desacetylase
BMD	Becker Muscular Dystrophy	iNOS	Nitrique oxyde synthase inductible
Ca ²⁺	Calcium ion	iPSC	Induced pluripotent stem cells
CaM	Calmodulin	IRES	<i>Internal Ribosome Entry Site</i>
Cas9	<i>CRISPR associated protein 9</i>	K19	Keratine 19
CH	Calponin homology domain	kb/Mb	kilo/megabase
CK	Creatine kinase	kDa	kiloDalton
CREB	C-AMP Response Element-binding protein	LBD	<i>Lipid binding domain</i>
CRISPR	<i>Clustered Regularly Interspaced Short Palindromic Repeats</i>	LNA	<i>Locked Nucleic Acid</i>
DAPC	<i>Dystrophin Associated Protein Complex</i>	lncARN	long noncoding RNA
del45-55	exons 45 to 55 deletion	MD1	MD1 Microdystrophin
DHPR	dihydropyridine receptor	<i>mdx</i>	<i>X-linked muscular dystrophy</i>
DMD	Duchenne Muscular Dystrophy	MHC	<i>Myosin heavy chain</i>
<i>DMD</i>	Dystrophin gene	miRNA	micro-RNA
dNTP	desoxyribonucleotides	miR	micro-RNA
domaine WW	domaine with 2 tryptophans	MLC	<i>Myosin light chain</i>
domaine ZZ	zinc finger domain	mtNOS	Mitochondrial nitric oxide synthase
DTT	Dithiothreitol		

myomiR	muscular micro-RNA	qPCR	quantitative PCR
NADH	Nicotinamide adenine dinucleotide	RISC	<i>RNA-induced Silencing Complex</i>
NADPH	Nicotinamide adenine dinucleotide phosphate	ROS	<i>Reactive oxygen species</i>
NHEJ	<i>Non-Homologous End-Joining</i>	RyR	ryanodine receptor
NMD	<i>Non-sense mediated RNA decay</i>	SAXS	<i>Small Angle X-ray Scattering</i>
nNOS	Neuronal nitric oxide synthase	SERCA	sarco/endoplasmic reticulum Ca ²⁺ -ATPase
O ₂	dioxygen	siARN	small interferent RNA
PABP1	<i>polyadenylate-binding protein 1</i>	SNP	<i>Single-nucleotide polymorphism</i>
PBS	<i>Phosphate Buffered Saline</i>	SOCE	<i>Store operated calcium entry</i>
PCR	<i>Polymerase Chain Reaction</i>	SRF	<i>Serum Response Factor</i>
PGC-1 α	<i>peroxisome proliferator-activated receptor gamma coactivator 1-alpha</i>	TA	<i>Tibialis Anterior</i>
Pi	Inorganic Phosphate	tcADN	tricyclo-DNA
PMO	phosphorodiamidate morpholino oligo	TLDA	<i>Taqman Low Density Array</i>
PPMO	PMO peptide	T _m	Hybridization temperature
polyA tail	polyadenylated tail	TRP	<i>Transient receptor potential</i>
		UTR	<i>Untranslated Transcribed Region</i>
		WGS	<i>Whole genome sequencing</i>
		WES	<i>Whole exome sequencing</i>

ÉPIGRAPHE

LE MYTHE DE SISYPHE

Toute la joie silencieuse de Sisyphe est là. Son destin lui appartient. Son rocher est sa chose. De même, l'homme absurde, quand il contemple son tourment, fait taire toutes les idoles.

Il n'y a pas de soleil sans ombre, et il faut connaître la nuit. L'homme absurde dit oui et son effort n'aura plus de cesse. S'il y a un destin personnel, il n'y a point de destinée supérieure ou du moins il n'en est qu'une dont il juge qu'elle est fatale et méprisable. Pour le reste, il se sait le maître de ses jours. À cet instant subtil où l'homme se retourne sur sa vie, Sisyphe, revenant vers son rocher, contemple cette suite d'actions sans lien qui devient son destin, créé par lui, uni sous le regard de sa mémoire, et bientôt scellé par sa mort. Ainsi, persuadé de l'origine tout humaine de tout ce qui est humain, aveugle qui désire voir et qui sait que la nuit n'a pas de fin, il est toujours en marche. Le rocher roule encore.

La lutte elle-même vers les sommets suffit à remplir un cœur d'homme.

Il faut imaginer Sisyphe heureux.

Le mythe de Sisyphe. Essai sur l'absurde. (Albert Camus, 1942)

PRÉAMBULE

Duchenne and Becker muscular dystrophies (DMD/BMD) are X-linked disorders due to mutations in *DMD* gene. DMD patients represent the most severe and lethal phenotype and the BMD patients the less severe clinical phenotype with a normal life span. In clinical practice, there are a large number of patients that feature intermediate phenotypes highlighting a broad clinical spectrum range.

The central dogma of therapy development is to transform severe DMD patients in milder BMD patients. Despite several exon skipping approved drugs and micro-dystrophin replacement therapies in ongoing clinical trials, there is no cure to Duchenne muscular dystrophy. The current caveats in therapy development may be explained from one hand by the wide spectrum of clinical heterogeneity in this population. From the over hand, the complex patho-mechanisms associated to this disease are not fully elucidated.

To complete my medical background, I decided to undertake my PhD in a translational study that could cover both aspects. For my PhD project we identified two aims, first one corresponds to the study of phenotype heterogeneity in population of major interest for drug development, the BMD Δ 45-55 cohort and the second aim was to study new potential actors involved in the phenotype variability.

This manuscript consists in four chapters, the first one includes the introduction with an overview of the literature data of interest to this project, the second chapter is based on 2 first author articles presentations that are currently in the process of publication submission in peer reviewed journals, a third article that was already published were I have contributed during the first year of PhD and the fourth one, which is a review of a recent therapy study in Spinal Muscular atrophy patients that highlights a large phenotype variability in treated patients that is in line with the main subject of this work. The third and the fourth chapters are discussing the reported results and the perspectives of this project.

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CHAPTER I: INTRODUCTION

SKELETAL MUSCLE

Generalities

Human body mass is consisted from approximately 650 muscles including 570 striated skeletal muscles. There are three types of muscle described in the human body: the skeletal muscle, the smooth muscle and the cardiac muscle. The skeletal muscular tissue functions are involved in voluntary actions as movement, locomotion, maintaining posture but also heat production due to catabolic reactions that are associated with muscular activity. The smooth muscle can be found in digestive, respiratory, cardiovascular, renal and reproductive systems, are involuntary.

CELLULAR ORGANIZATION OF A MUSCLE FIBER

The muscle fibers have a unique microstructure that has specialized terminology. The main characteristics of the muscle fibers and also the muscle cells are the bundles-within-bundles structure. At the surface a tough layer of connective tissue, epimysium, is connected to muscle tendons at each end. It protects the muscles from friction against other muscles and bones¹. Within the epimysium are localized the fascicles that have up to >100 muscle fibers that are sheathed by the perimysium. At this level are localized the pathways for the nerves and blood vessels. Within the muscle fiber the bundles of protein filaments are called myofibrils of a tubular shape (Figure 1). The myofibrils are complex strands of myofilaments organized together into repeating units called sarcomeres, the contractile units of the cell. The sarcomeres are composed of protein filaments that slide along each other during a muscle contraction or relaxation. There are three types of myofilaments units composed as follows by: the thick myofibrils called myosin (A line), the thin filaments composed by actin (Z line) and the elastic filaments composed by the titin protein. Myosin protein is composed by a long tail and a globular head which binds to actin and also to ATP and provides energy for the muscle movement (Figure 1B)^{2,3}.

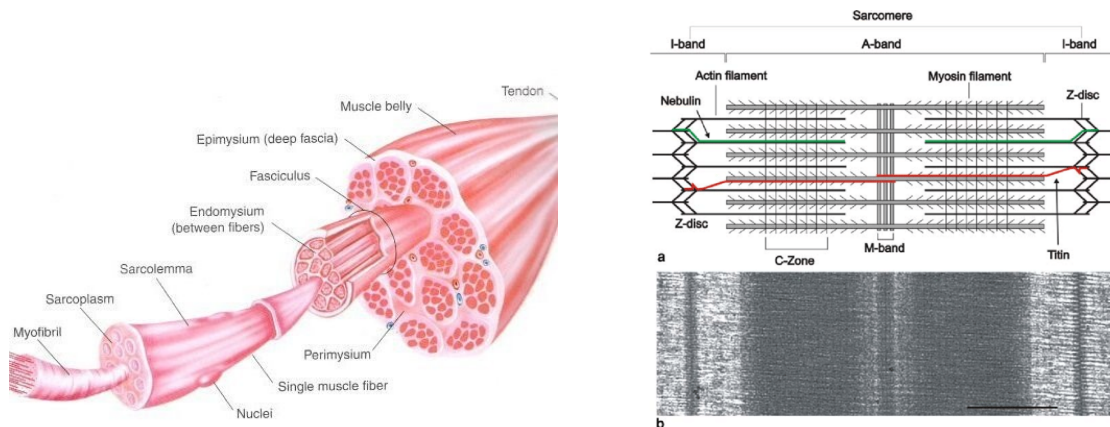


Figure 1 Muscle fiber and sarcomere structures.

(A) Bundle within bundles muscular fiber, (B) The basic contractile unit of skeletal muscle, the sarcomere, is composed of thin and thick filaments predominantly composed of actin and myosin, respectively. Thin filaments of adjacent sarcomeres are anchored at the Z-disk, which defines the lateral borders of the sarcomere. Myosin has a long, fibrous tail and a globular head, which interacts with actin to produce muscle contraction. Adapted from Luther, P. 2009 and Rand, C 2020

Into the muscle fibers the sarcoplasmic reticulum (SR) is characterized by a network of tubules that extends throughout muscle cells and wrapping around the bundles-within-bundles structure of muscle fibers. The SR terminal cisternae and the sarcolemma T-tubules form the triad and act as a major actor in the contraction generation (Figure 2)^{4,5}. The SR is a membrane bound structure and plays the role of Calcium ions storage by a buffer protein the calsequestrin assisted by two other proteins the Triadin and Junctin found within the SR membrane. The calcium ions release occurs via the terminal cisternae through a ryanodine receptor (RyR) that is tissue specific (RyR1 in skeletal muscle, RyR2 in cardiac muscle and RyR3 in the brain)⁶.

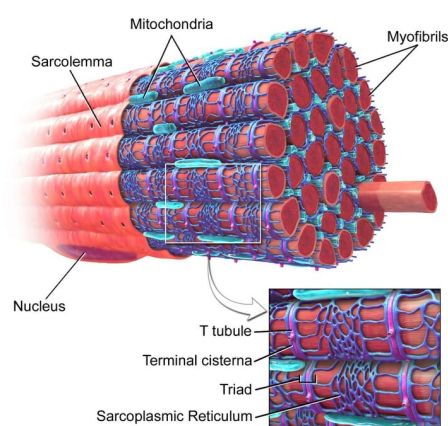


Figure 2 Sarcoplasmic Reticulum (SR) and Triad partners.

Adapted from Laporte, 2011

MYOGENESIS

Myogenesis is the process of generating muscle and is separated in developmental (embryonic and fetal) myogenesis and adult regeneration.

DEVELOPMENTAL MYOGENESIS

This highly complex function is divided in several distinct phases⁷. During embryogenesis the prepatterned embryo will develop ectoderm, mesoderm and endoderm. The mesoderm will separate in the course of development and will result into three layers: the paraxial, intermediate and lateral with respect to position from midline. Muscles will derive from the paraxial mesoderm and will divide into somites. Somites will receive signals, including Wnts, Sonic hedgehog (Shh), BMP-4, insulin-like growth factor - 4(IGF-1) and noggin, from the neighboring tissues that will induce primordial muscle precursors cells to be committed in a myogenic fate and to differentiate into mature muscles^{8,9}. The somites will have three divisions, the sclerotome, dermatome and the myotome. After the formation of connective tissue frameworks, myoblasts (muscle progenitor cells) migrate to the rest of the body to form other muscles¹⁰⁻¹². The embryonic myogenesis and adult muscle regeneration have many similarities as common transcription factors and signaling molecules. The satellite cells are closely related to the progenitors of somatic origins as the muscle resident myogenic progenitors will initially proliferate extensively but will enter in quiescence as satellite cells once the muscle matures^{10,11}. During development, the extrinsic regulatory system directs spatio-temporally distinct fates of self-renewal or differentiation to myogenic precursors¹² (Figure 6).

Developmental myogenesis is regulated by the myogenic regulatory factors (MRF), that are able to induce myoblast traits in nonmuscle cell lines. Shh (from the notochord), and Wnt1/3, Ant11 and IGFs (from dorsal neural tube) signaling are regulating the early expression of Myf5, the first MRF expressed during embryonic development^{8,13,14}. But the *Myf5* factor does not act alone as it was revealed that this MRF is not expressed in all myoblasts and the myogenesis was possible by a *MyoD* expressing lineage. As suggested by the phenotypic studies the Myf5 and MyoD act upstream of *Myogenin* and *MRF4* factors^{15,16}. Both, *Myogenin* and *MRF4* are involved in the differentiation process and the expression of myotube specific genes. As per

this temporal classification the paired-homeobox transcription factors *Pax3* and *Pax7* appear to be expressed earlier than *MyoD*. The loss of the *Pax3* lineage is embryonically lethal and in contrary the ablation of *Pax7* lineage will result in defects in later stages of development and result in fewer myofibers in the limbs at birth^{11,17,17,18}.

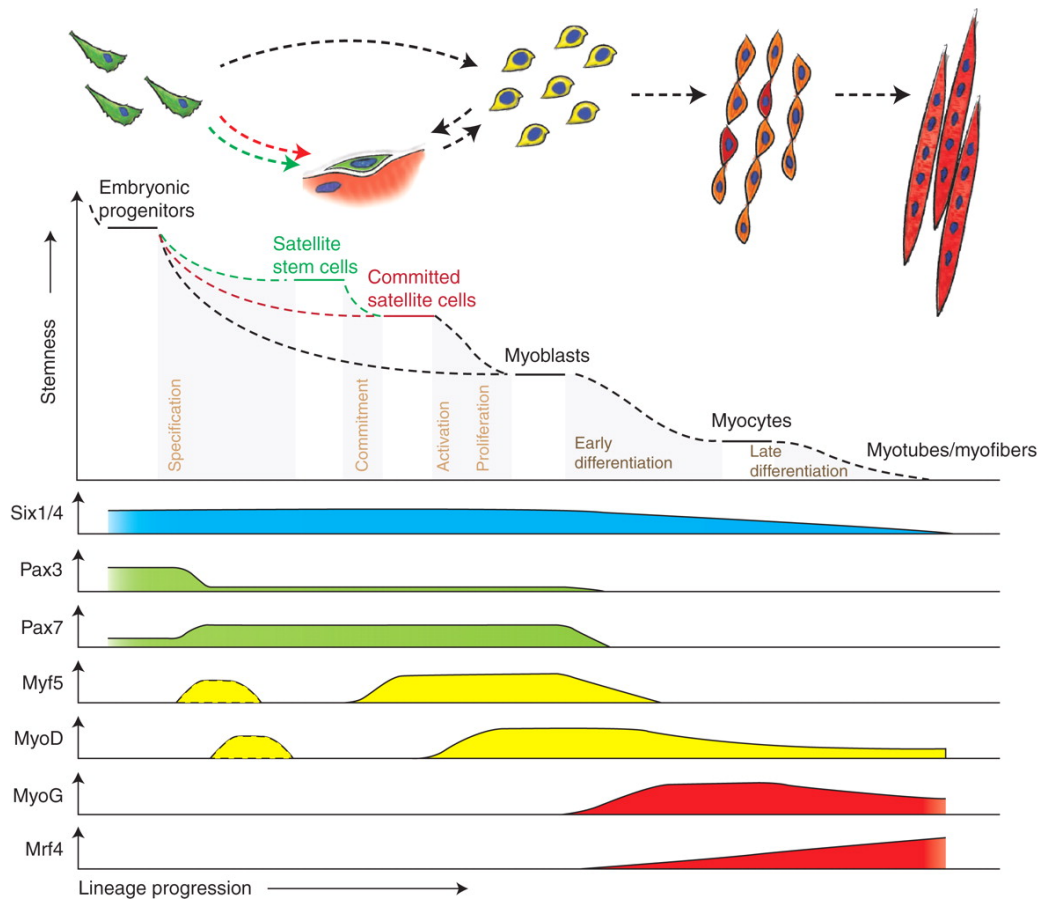


Figure 3 Hierarchy of transcription factors regulating progression through the myogenic lineage.

Adapted from Betzinger, F 2012

Muscle progenitors that are involved in embryonic muscle differentiation skip the quiescent satellite cell stage and directly become myoblasts. Some progenitors remain as satellite cells in postnatal muscle and form a heterogeneous population of stem and committed cells. Six1/4 and Pax3/7 are master regulators of early lineage specification, whereas Myf5 and MyoD commit cells to the myogenic program. Expression of the terminal differentiation genes, required for the fusion of myocytes and the formation of myotubes, are performed by both myogenin (MyoG) and MRF4^{12,14}.

REGENERATION OF ADULT SKELETAL MUSCLE

The regeneration refers to the process of renewal, restoration and tissue growth that is caused by a stress or damage. One of the major actors of muscle regeneration is played by the muscle stem cell known as satellite cell which resides between the basal lamina and sarcolemma of myofiber. The satellite cells are described as “dormant myoblasts that failed to fuse with other myoblasts and are ready to recapitulate the embryonic development of the skeletal muscle fibers when the multinucleate cell is damaged” in other words, they are mitotically quiescent until required for growth or repair. Recent data reported the role of the Notch signalling in the regulation of cellular quiescence in adult muscle stem cells¹⁹⁻²¹. Once activated the satellite cell will follow two pathways depending on MyoD activity and self-renewal guaranteeing the maintenance of a pool of Pax7 positive cells¹¹. Proliferation to differentiation transition involves the differential expression of proliferative-associated genes with the downregulation of the proliferation specific ones and cell-cycle withdrawal.

This asymmetrical expression of proliferation or differentiation associated genes involves the Notch, Numb and Wnt pathways¹¹. The myogenic differentiation has been demonstrated to be orchestrated by Numb family by inhibiting Notch signalling in the daughter satellite cell. In differentiation onset the Wnt will take over the Notch signalling in myogenic progenitor and this crosstalk occurs via GSK3beta^{22,23}.

In addition to the protein regulation of these complex processes, the non-protein coding regulators gain attention and place. Non-coding RNA are increasingly described as active regulators of proliferation and differentiation processes²⁴⁻²⁶. Also, some long non-coding RNA (lncRNA), defined by the length > 200nt ncRNA can regulate gene expression both in cis or trans. This is the case of the lncRNA Yam-1 that is downregulated upon differentiation and will recruit miR-715 in order to target Wnt7b and act as a myogenesis inhibitor^{27,28}. A different study reported the role of the lncRNA Six 1 in cell proliferation and muscle growth function of the chicken *Six1* gene²⁹. The MUNC lncRNA localized upstream MyoD can increase endogenous MyoD, MyoG and Myh3 mRNA. In addition to gene regulation, some lncRNA can also bind to proteins in order to regulate the muscle development and atrophy thus becoming interesting candidates in myogenesis and regeneration studies^{30,31}.

Thus, muscle development and homeostasis through regeneration depends on complex regulatory networks. Their perturbation is involved in structural and functional changes that occur during muscle atrophy/hypertrophy and regeneration. Therefore, defective regeneration is involved in various muscular diseases including muscular dystrophies, sarcopenia and cachexia. The identification of new components controlling muscle differentiation and regeneration could clarify the molecular pathogenic mechanisms in different diseases and potentially help the development of new therapies.

EXCITATION-CONTRACTION COUPLING

The contractile function of the muscle is ensured by the excitation-contraction coupling, a process by which a muscular action potential causes the contraction of myofibrils resulting in concentric and eccentric muscle contraction³² (Figure 3).

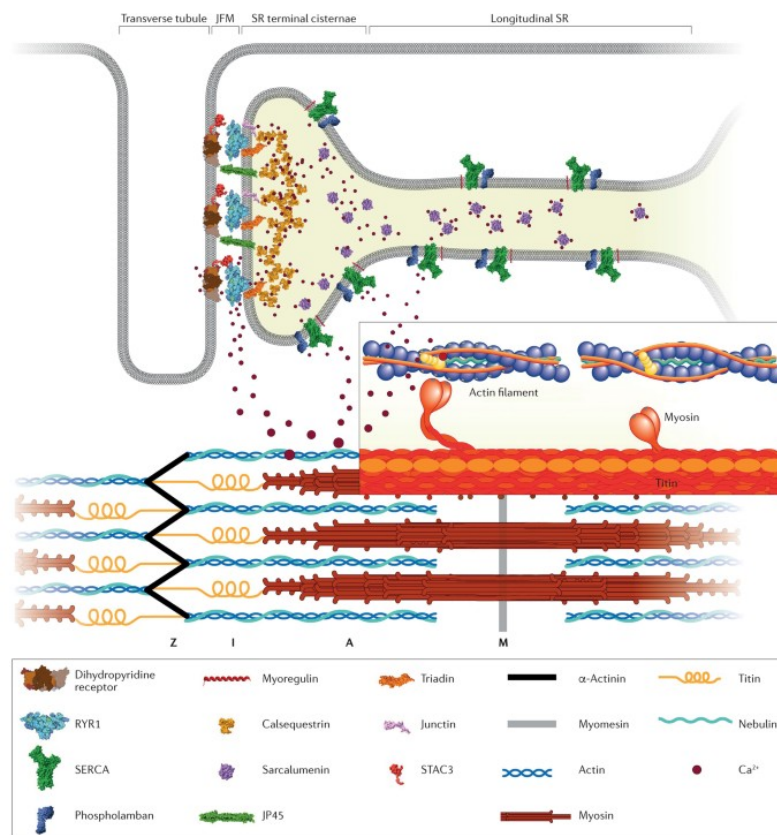


Figure 4 SR Ca²⁺ release and myofibrils contraction illustration.

Adapted from Jungbluth et al., 2018

The skeletal muscle contractions are neurogenic as they are initiated by a synaptic input from the motor neurons, followed by a release of the neurotransmitter acetylcholine into the neuromuscular junction. The process of the release and binding of the acetylcholine to the post-synaptic nicotinic acetylcholine receptor will make a change in the conformation of this receptor and allow an influx of sodium ions that will initiate a post-synaptic action potential. This action potential will travel along the sarcolemma to the T-tubules of the Sarcoplasm and will reach the SR^{4,33,34}.

Dihydropyridine (DHPR) receptors, L-type calcium channel Cav1.1 voltage sensor localized on sarcomere T-tubules mediate detection of changes in membrane potential (depolarization) and will activate the RyR1 receptor leading to a massive release of the calcium ions from the SR in the cytoplasm. Calcium will bind to troponin allowing the actin-myosin binding and myosin heads evacuate power stroke and allow to actin filament to slide toward the center of the sarcomere. The decrease of free cytosolic calcium will unbind the remaining calcium from troponin and will result in myosin head release and the return to the relaxed position. Dynamic regulatory process between partner proteins as calsequestrin, triadin, junctin and the calcium concentration assure the fine tuning of the RyR1 receptor³⁵.

THE DYSTROPHIN-ASSOCIATED PROTEIN COMPLEX (DAPC)

The dystrophin-associated protein complex (DAPC) is one of the most important structure allowing the maintenance of fiber integrity during muscle contraction. This multidimensional structure spanned the different levels of the cellular localization (Figure 4): extracellular (α -dystroglycan); transmembrane (β -dystroglycan, sarcoglycans, sarcospan); cytoplasmic (dystrophin, dystrobrevin, syntrophins, neuronal nitric oxide synthase). Indeed, the DAPC complex has two major roles, the first one as a cornerstone of the membrane stability during muscle contraction and the second one as a scaffold for various signaling and channel proteins^{36,37}.

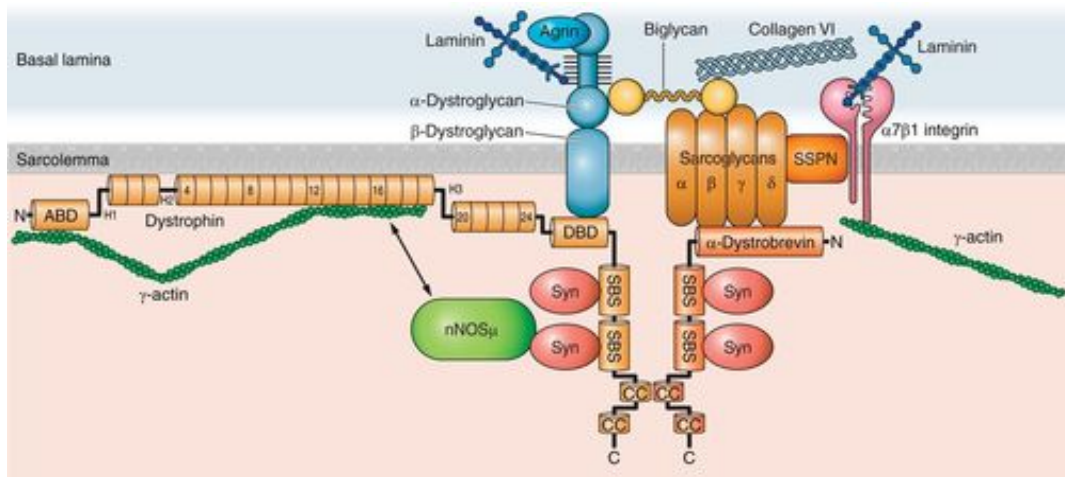


Figure 5 DAPC structure.

The DAPC is a multimeric protein complex that connects the intracellular cytoskeleton of a myofiber to the ECM, which is composed of laminin, collagen, and other proteins. Adapted from F. Rahimov 2013

At the extracellular level, the α -dystroglycan act as receptors for the ligands as laminin and participate to the muscle cell adhesion to the basal lamina. The α -dystroglycan is glycosylated on serine residues (O-glycosylation pathway) and large variety of genes, involved in α -dystroglycan glycosylation as *POMT2*, *POMT1*, *FKTN*, *FKRP* results in a variety of limb girdle associated dystrophies as LGMD2I, K, N and O. Also, the mutation in genes as *LARGE*, *GTDC2*, *POMK* have been identified to cause a large spectrum of congenital muscular dystrophy by interfering in the α -dystroglycan glycosylation pathway. Altogether this large subset of muscle diseases referred as dystroglycanopathies are featuring reduced dystroglycan glycosylation and a high variability in the phenotype presentation ranging from mild congenital muscular dystrophy to dramatic and lethal condition such as brain and eye anomalies. Interestingly, the mutation of the dystroglycan gene itself was identified only recently, as a missense mutation (T192M) that disrupts dystroglycan binding to laminin. This data suggests a vital role in early embryonic development beyond muscle tissues as for example the dystroglycan null mice are embryonic lethal³⁵.

The Sarcoglycan is composed with four single-pass transmembrane proteins: α -sarcoglycan, β -sarcoglycan, γ -sarcoglycan and δ -sarcoglycan³⁸. A single mutation in the genes encoding for these proteins leading to the loss of the transcript open reading frame, affects the stability and the role of the entire complex resulting in limb girdle muscular dystrophies as LGMD2C-F.

The sarcoglycans complex and at the cytoplasm level, the dystrophin protein both interact with β -dystroglycan.

In the cytoplasm syntrophin and α -dystroglycan associate with dystrophin and anchor nNOS to the sarcolemma. Indeed, the specific subsarcolemmal localization of dystrophin assure the membrane maintenance by interaction with the DAPC complex^{37,39}.

DYSTROPHIN

DYSTROPHIN PROTEIN

Dystrophin protein belongs to the DAPC. It is a large (427kDa) sub-sarcolemmal protein that connects cytoskeleton of muscle fibers to the extracellular matrix. The main role of dystrophin is to act as a stabilizer of muscle fiber during the movement. The absence of this protein destabilizes the sarcolemma and result in damage during the contractions. As a structural protein, dystrophin, acts as a scaffold to the assembly of multifunction DAPC complex but also is involved in cell signaling function.

Dystrophin displays four main functional domains, the actin binding amino-terminal (ABD1), the central rod domain, a cysteine-rich domain and a carboxyl-terminus^{35,37} (Figure 5).

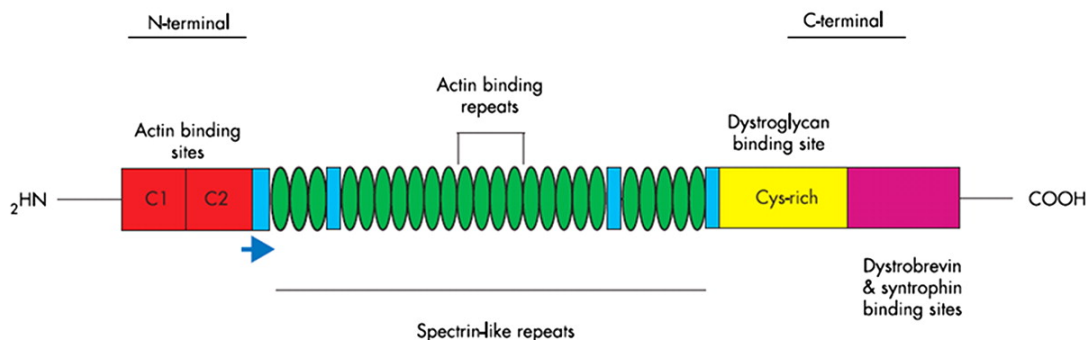


Figure 6 The dystrophin protein.

Dystrophin protein has four functional domains including the N-terminal, a long middle rod, cysteine-rich, and C-terminal domains. The central rod domain consists of 24 spectrin like repeats arranged head-to-tail and interspersed by four flex. Adapted from Yilmiz, A 2011

The ABD1 has two calponin homology domains (CH1 and CH2) that will connect dystrophin to the actin and anchor it to the costamere proteins as cytokeratin 19 (K19)⁴⁰. The central rod domain has 24 spectrin repeats in a flexible rod shape supposed to assure the shock absorber function. The rod domain has a second ABD2 and form with ABD1 a lateral association with the actin filaments. The spectrin-like repeats 8-9 are connected to PAR-1b and phosphorylates sites in this region, additionally the repeats 20-24 are connected to the microtubule network⁴¹. Interestingly, in vivo experiments revealed the sarcolemmal targeting of nitric oxide synthase (nNOS) an important partner protein was dependent on the spectrin-like repeats 16 and 17 within the rod domain of dystrophin protein. This data suggest that the role of spectrin-like repeats is more than shock absorber and likely serve as a scaffold for the signaling proteins (see details in section 5).

There are four short proline-rich spacers between the 24 spectrin-repeats⁴². At the end of the hinge 4 a WW domain that act as a protein-protein anchor⁴³. Together, the WW with the EF-hands motifs bind the carboxy-terminus to the β -dystroglycan⁴⁴⁻⁴⁶. The cysteine rich domain contains zink finger (ZZ) cysteine reach domain that is the link of the dystrophin to the calmodulin^{47,48}. The carboxy-terminal (CT) domain binds to the syntrophins and dystrobrevin^{39,49,50}.

There are three filamentous structures as F-actin, microtubules and intermediate filaments that are associated to the DPAC and involved in the dystrophic pathology process by their association to the dystrophin protein. For example, the microtubules have a dual structural and signaling function as there are associated to the ROS production in dystrophic muscle. The rod domain of dystrophin is supposed to be acting as a guide for the arrangement of the microtubule lattice⁴¹. The F-actin and the intermediate filaments represent an important element of link to the DPC complex.

CHARACTERISTICS OF THE *DMD* GENE

The *DMD* gene is the second largest gene after the *TTN* (*titin*) gene and is localized on X chromosome at Xp21 locus and is approximative 2.4 Mb (Figure 7). It was first described by

Hoffman et al, 1986 and its protein description came a year later⁵¹. The gene is composed of 79 exons and results in a full length 427 kDa protein. The transcription of *DMD* gene is controlled by 7 promoters, three independent ones, reflecting the tissue distribution in brain (B), muscle (M), Purkinje (P) and four internal (R) for retinal, B₃ for Brain 3, S for Schwann cells, and G for General^{36,52}. There are three full-length tissue-specific isoforms that are expressed in brain (Dp427b) (cerebral cortical, hippocampal), muscle (Dp427m) and Purkinje fibers (Dp427p) and have each a different promoter and first exon. Splicing of the internal promoters (from a unique exon) will result in 5 shorter isoforms. The remaining shorter isoforms are expressed in the retina (Dp260), the Dp140 expressed in the brain but also in the retina and kidney and it starts the exon 45 and the Dp116 (Schwann cells). The shortest isoform, the Dp 71, is ubiquitous⁵².

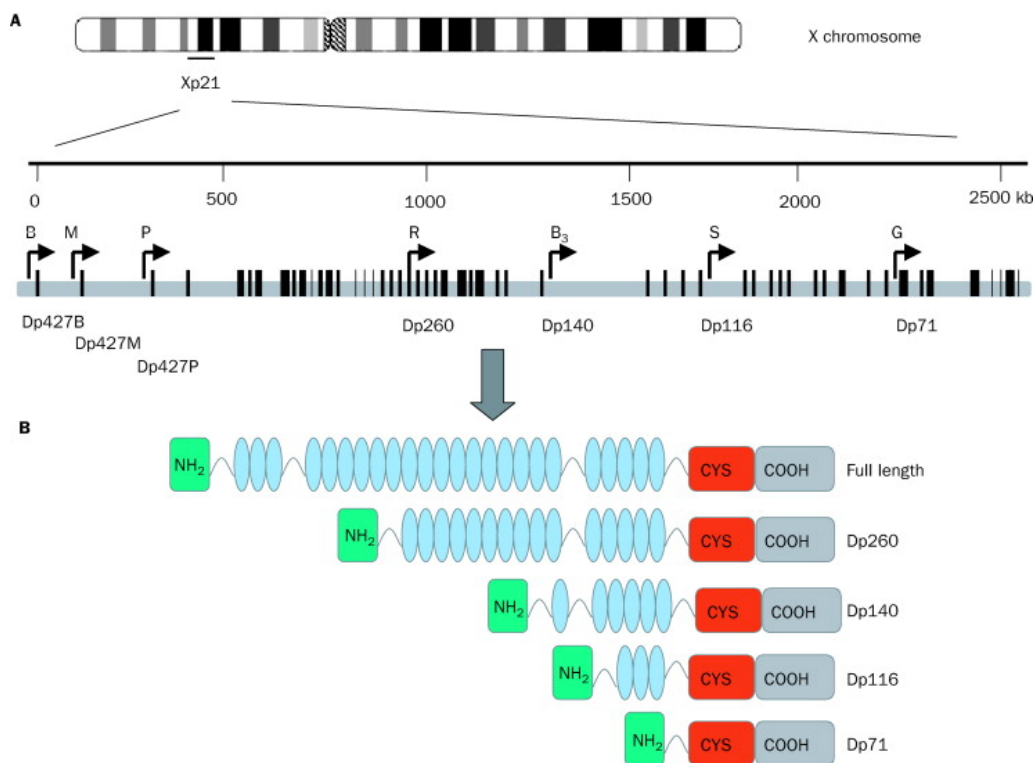


Figure 7 Schematic illustration of X chromosome and the *DMD* gene.

Black arrows indicating the promoter localization of each dystrophin isoform. (B) Dystrophin isoforms classified by size length. Adapted from Muntoni, F 2003

The large size of this gene can be explained not only by the largest exon number but also by the intron's length. About 99% of *DMD* gene is represented by its non-coding sequences as the transcript is only 14kb long and the ORF (open reading frame) is 11kb. Indeed, two very long

introns are present in the gene: intron 44 (248kb) and intron 55 (120kb) while the others are 2.3-5.4 BP⁵³(Figure 8 B).

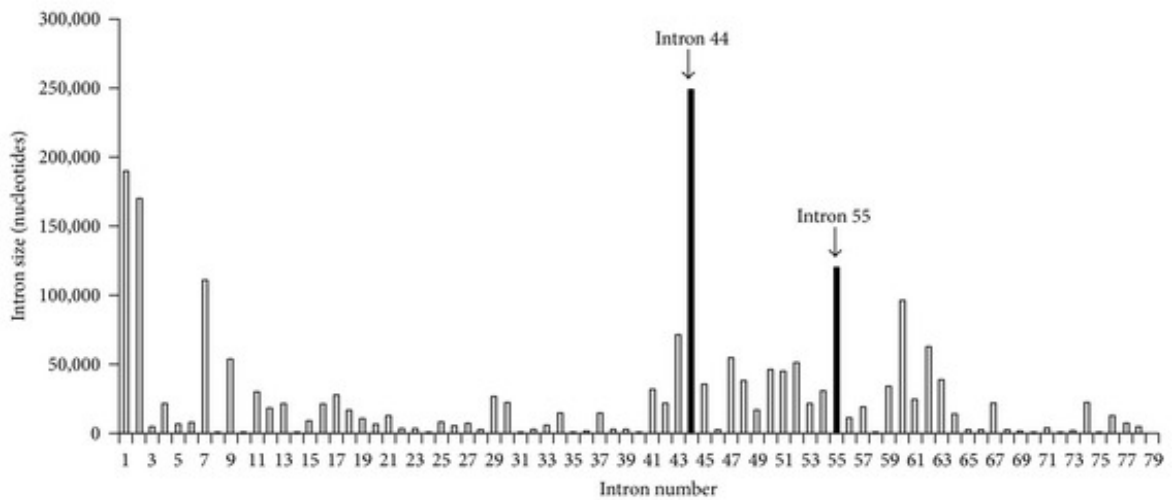
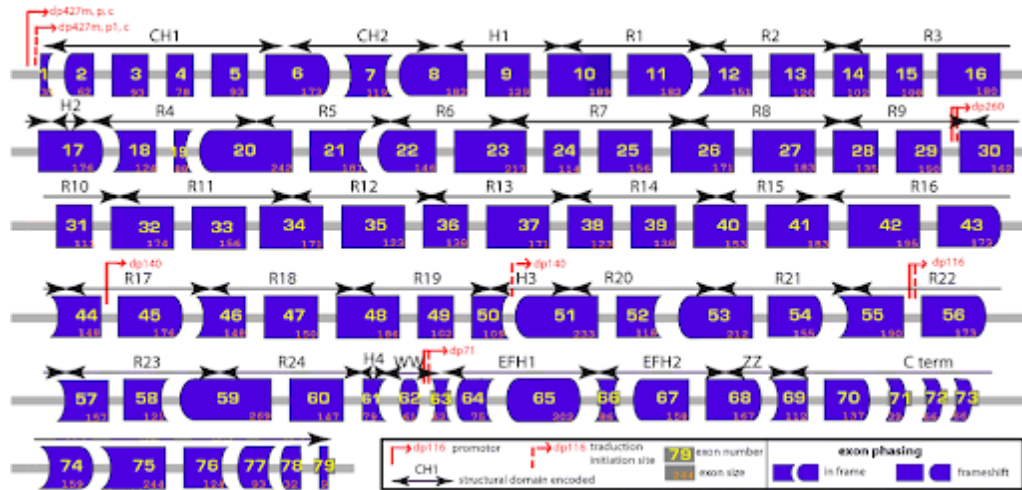


Figure 8 DMD exons and introns scheme illustration.

(A) Exons are illustrated in blue boxes and introns grey line. Red arrows indicating dystrophin isoform promoters. Black arrows illustrating the dystrophin protein structure. (B) DMD gene introns illustration. Adapted from *e-dystrophin et Aoki et al.*

There are more than 7000 different known mutations and a high spontaneous mutations rate with in 2/3 of cases maternally inherited mutations and in 1/3 *de novo* mutations^{54,55}. In the large mutational spectrum, the 2/3 of total mutations are deletions and the rest are duplications, insertions, point mutations and splicing mutations. The type of mutation and more precisely the interruptions of the reading frame and subsequently the protein production is predictive of the clinical phenotype in the majority of cases. Monaco *et al.* 1988 and Koenig *et al.* 1989 have described the reading frame mutation theory separating BMD and DMD^{56,57}.

As per Monaco rule, if a mutation leads to an out-of-frame shift and results in a premature stop codon that will not allow the production of a functional dystrophin protein, the patient will be associated with a DMD phenotype. In contrary, in BMD the in-frame mutation preserves a shorter but functional protein and is associated with a lighter clinical phenotype^{50,58,59}.

BMD and DMD are X-linked recessive disorders. Despite the X-linked recessive transmission and thus largely affecting boys/men, there are also affected females due to the X-inactivation. It has been described that 1/3 of females carrying DMD mutation are presenting clinical symptoms as described in BMD and very few cases as DMD type due to the mutations in the both gene alleles^{60,61}. Several studies have identified two mutational hotspots at exon 3-9 and exon 45-55. Interestingly, the multiple exons 45-55 deletion are in-frame deletion and the described patients with this mutation are described as having a mild phenotype⁵⁰.

The detailed study of *DMD* gene spectrum of mutation in DMD patients revealed that 61% of patients present large deletions and 13% duplications and in 26% point mutations. As for BMD patients in 81% the mutations are deletions and only 6% duplications and finally in 13% point mutations^{55,62}. It is known that DMD/BMD can results from either inherited or spontaneous mutations in the *DMD* gene^{63,64}.

Interestingly there are some exceptions to the Monaco rule, for example, in patients with milder phenotypes who had out-of-frame deletions of exons 3–7 or patients with deletions that did not disrupt the reading frame who exhibited more severe phenotypes^{58,63}. Moreover, Flanigan et al reported a particular BMD founder allele (c.9T>G; p.Trp3X) that escaped from the reading frame rule. Indeed, despite a nonsense mutation resulting in absent protein translation the patient featured mild dystrophinopathy and the muscular biopsy detected dystrophin protein expression at relatively high level with a minimally decreased size. Authors reported that the

amelioration of disease severity result from alternative translation initiation beginning in DMD exon 6 that lead to the expression of a highly functional N-truncated dystrophin by using a glucocorticoid - inducible internal ribosome entry site (IRES) within exon 5^{65,66}.

BMD WITH HOTSPOT DELETION OF EXONS 45-55 (BMDDEL45-55)

We reported here a large mutational *spectrum* of the *DMD* gene. Indeed, not only there are an increasing number of reported exceptions to the reading frame rule but also several regions in the *DMD* gene have a higher risk for mutations.

IN-FRAME VERSUS OUT-OF-FRAME

Deletions can occur anywhere within the *DMD* gene, but a mutations hotspot has been described localized between exons 44 and 55, the longest introns of the gene^{62,67}. Deletion spanning exon 45 to exon 55 (del45–55) is associated with very mild or even asymptomatic cases^{68,69}. This deletion removes spectrin repeats 17 to 22, and this truncated dystrophin lacks part of the binding sites for neuronal nitric oxide synthase (nNOS), mediated by spectrin repeats 16 and 17. Although generally mild, variations of disease severity exist between patients carrying del45–55, and this variable phenotype correlates with nNOS sarcolemma localization⁷⁰. In a study of 12 patients with del45–55, the mild/asymptomatic group had mainly sarcolemma nNOS, while the more severe group exhibited an exclusive cytosolic nNOS⁷¹.

ABSENT VERSUS PRESENT TRUNCATED PROTEIN

Since the clinical description of DMD/BMD and the discovery of the *DMD* gene, the development of dystrophin-specific antibodies led to the recognition of the dystrophin expression as a diagnostic biomarker. From the first reported studies until now, the postulate of

the lack of the 427 kDa dystrophin protein in muscle as a positive DMD diagnostic argument remains still actual. However, despite a large number of research studies at this time, the amount of dystrophin needed to assure clinical benefit remains uncertain^{71,72}.

As stipulated in literature data very low level dystrophin was detected in DMD patients. It was assumed that the presence of revertant fibers altogether with the presence of minor in-frame alternatively spliced mRNAs could explain the positive detected dystrophin^{52,63,73}.

Literature data report dystrophin levels below 10% in DMD patients and very severe BMD patients⁵⁸. In a recent study, our team reported that BMD patients had a range of dystrophin level with the lowest above 60%⁷⁴. Different authors reported patients with exon 45–47 deletion with dystrophin levels varied from 13% to 76%, however no correlation between dystrophin protein level and disease severity was found⁷⁵. Similarly, threshold effects were observed in another study comparing dystrophin levels in out-of-frame to those in in-frame deletions within the exon 42–49 region⁷⁶. In regard to recent FDA/EMA approved therapies by exon skipping, it is coherent that even low level of truncated dystrophin can be beneficial (EXONDYS 51).

As mentioned above in BMD patients, the dystrophin in-frame transcripts allow the production of a shorter but functional dystrophin. Despite that BMD patients have a high clinical variability. Many dystrophin quality and quantity studies tempted to define disease evolution dichotomy between DMD and BMD; on top of the variability of quantity and probably quality of present dystrophin, it should be underlined that this protein is present in BMD patients since birth in opposition to DMD patients⁷⁷.

Dystrophin structure involvement in clinical heterogeneity was analyzed in BMD patients carrying different in-frame deletions in the mutational hot spot of the *DMD* gene. Clinical phenotype was correlated with modelling and biochemical data and found that the structural characteristics of dystrophin at the deletion site are strongly associated with phenotype severity in BMD patients. Precisely, BMDdel45–48 and del45–51 patients exhibit structural features similar to wild-type protein at the deletion site have a slower disease progression compared with del45–47 or del45–49 deletions which both lead to profound structural modifications of dystrophin⁴⁵. Similar data were reported for del45-47 of 45-48 patients in a different study that reported BMD phenotype in 97%⁷⁸.

Moreover, recent studies revealed long non-coding RNA (lncRNA) sequences localized in intron 44 and 56. The expression profiles of these lncRNA were inversely correlated with muscle dystrophin expression in DMD female carriers suggesting a negative relationship between lncRNA and dystrophin mRNA⁷⁹.

These data suggest that in addition to the dystrophin quantity, the structural difference of the resulting protein but also the presence of regulatory sequences are influencing the resulting clinical phenotype.

DYSTROPHINOPATHIES

CLINICAL PRESENTATION

Muscular dystrophies are genetic conditions characterized by muscle waste with progressive evolution of weakness and atrophy. The Duchenne, Becker and X-linked muscular dystrophies are due to mutations in the same gene. All these related conditions affect skeletal and cardiac muscles.

DUCHENNE MUSCULAR DYSTROPHY (DMD)

DMD is a lethal progressive genetic, X-linked disorder due to mutations in the DMD gene and resulting with the absence of functional dystrophin protein. DMD is an X-linked recessive condition affecting 1 in 5000 newborn boys^{45,64,80-83}. As described earlier, the dystrophin protein is a large structural protein that connects the cytoskeleton of muscle fibers to the extracellular matrix and one of its known functions is to stabilize the muscle fiber during the movement. The absence of this large protein is causing muscle fibers damage and results in inflammation and muscle fiber regeneration inhibition. Progressively inflammation-necrosis cycles lead to the replacement of the muscle with fibrotic and adipose tissue.

The clinical presentation is well described, starting in the early childhood at the age of 2-3 years with a global developmental delay in the first place^{76,83-85}. The personal history of affected boys is characterized mainly but not exclusively with a delay in the walking acquisition (> 18 months), progressive walking impairment with a tiptoe walk due to ankle contractions and a classic Gowers sign⁸⁶. The gradual loss of muscle tissue results in loss of ambulation approximately at the age of 12 years, despite the supportive corticosteroid therapy^{84,85,87-89}. Progressively the upper limbs are affected with respiratory and cardiac complications resulting in a limited life expectation and premature death in the fourth decade of life^{84,90,91}.

The actual standards of care recommend the corticosteroids therapy in the majority of patients under close monitoring for the side effects. This treatment delayed the age of the loss of ambulation and help to maintain the muscle force. The cardioprotective therapy, the

noninvasive ventilation aside with the cough assist, nutritional management with gastrointestinal tube feeding allowed to improve the quality of life^{83,84}.

X-LINKED CARDIAC DYSTROPHY (XLCD)

A particular place in the spectrum of BMD phenotype presentation are the cardiac myopathy without skeletal muscle symptoms, the age of onset is between 20 or 30 years in young men with a rapid evolution and sometimes severe presentation because of the lack of skeletal muscle signs. In female carriers the onset is later, around 40 years old and the evolution of slowly progressive. This presentation underlines the importance of the family genetic counseling in all newly diagnosed families.

BECKER MUSCULAR DYSTROPHY (BMD)

GENERAL DESCRIPTION

Becker muscular dystrophy (BMD) is a part of a dystrophy disorders caused by mutations in the *DMD* gene with a large spectrum of phenotype presentation. The BMD is caused by “in-frame” mutations in DMD gene that results in a partial or truncated dystrophin protein.

BMD was first described by Emile Becker in 1953 as a variant with a mild severity form of the Duchenne muscular dystrophy (DMD). The clinical presentation is very heterogenous ranging from asymptomatic or pauci-symptomatic forms with increased level of CPR (creatine-phosphokinases) or with skeletal muscles cramps with or without associated myoglobinuria. The severe clinical presentation can have a similar phenotype as in DMD presentation with severe impairment of skeletal muscles or have a mainly cardiac presentation.

BMD is a rare disease, affecting 1:11,500–19,000 males^{64,92}. The large range of the clinical presentation's spectrum with the presence of mild clinical phenotypes can explain the difference in the incidence of BMD with other muscular dystrophies, as DMD which is 5-6 times more frequent. Cardiac failure is the major complication in both DMD and BMD^{64,93}. In BMD patients with poor skeletal muscle phenotype rare cases of emergency heart transplant were reported. Classically BMD patients have a slowly progressive skeletal muscle impairment. The actual standards of care, especially concerning the cardiac follow-up and treatment improved quality of life in BMD patients.

CLINICAL PRESENTATIONS

THE CLASSIC PRESENTATION

Young adult male consulting the general physician for the skeletal muscle complains characterized by slowly progressive muscular deficit of the pelvic muscle groups, an impressive bilateral hypertrophy of calves and elevated CPK and liver enzymes^{85,91,94,95}. The family history can be positive with a recessive X linked transmission pedigree. The first clinical signs appear in the early teens, with a slow progression of the skeletal muscle symptoms. The skeletal muscles symptoms start with the lower limb's weakness with an association in walking fatigue and/or deficit. The walking injury is associated in some cases with an effort intolerance and/or pain and/or fatigue. The first involved muscle groups in lower limbs include the quadriceps and the hip flexors with a secondary involvement of upper limb, more particularly with biceps and wrist extensors. The muscular deficit is characterized by a particular walking pattern with a tiptoe walk, waddling gate and positive ankles retractions. Similar to the DMD boys, the patients have Gowers's maneuver, which is characterized by a muscular deficit when rising from the floor. Another common feature with DMD presentation is the calf bilateral hypertrophy, commonly explained by the ongoing process of fibrosis and oedema. The difference between the DMD/BMD clinical presentation remains clinical and based on the disease progression in the skeletal muscle involvement. The decision-making criteria is based on the lower limbs muscular deficit evaluation looking for the age of loss of ambulation (LoA) and/or wheel chair dependence: < 13 years old for the DMD phenotype and > 16 years for the BMD clinical presentation, with several intermediate phenotype presentation (13-16 yo). The link between the age of first clinical signs and the progression of the disease remain unclear. The current available data implies a slow progressive evolution for the patients with first clinical signs >30 years old.

In addition to the skeletal muscle presentation, BMD patients have an association with cardiac dilatative cardiomyopathy^{64,93}. The cardiac injury is characterized by the left ventricular ejection fraction (LVEF) <45%. The cardiac presentation can be also variable, in some patients with a classic presentation of dilated cardiomyopathy, in others the cardiac injury is slow and marked by a hypokinetic cardiac function associated or not with fibrosis of the left lower lateral cardiac territory. The cardiac failure remains common in the patients with absent cardiac follow-up and preventive treatment. Some patients have also an association of moderate

conduction impairment, and extrasystoles, the risk of the sudden death and a ventricular tachycardia or ventricular atrial block (VAB) remain very low. The prevalence of cardiomyopathy is age dependent and estimated between 30-40%, 15% with the age of onset before 20 yo and 55% after 40years old. BMD patients can have the association of the central nervous systems impairment, more commonly described in the infantile clinical presentation.

EARLY INFANCY PRESENTATION

BMD is commonly described as an adult disease. The detailed personal history can underline in some cases a developmental delay associated with a delay in walking acquisition and frequent falls in early childhood. The young boys present at clinical examination symmetric calf hypertrophy and low limb deficiency with various severity. The lower limb deficiency can be responsible for the tiptoe walking, ankle retractions and progressively the onset of the pelvic insufficiency. As mentioned above, the first clinical signs are not necessarily exclusively motor but also fatigue, effort intolerance, attention and hyperactivity disorders with or/without cognitive impairment. In some cases, autistic spectrum features are described in first intention with secondary skeletal muscle symptoms^{81,95}.

PAUCI-SYMPOMATIC PRESENTATION

The BMD is characterized by a very heterogeneous clinical presentation with several patients presenting isolated high CK level, myalgia, calf hypertrophy, cramps without any skeletal muscle deficit. These clinical presentations can be responsible for the delay in the diagnosis and represent a group of patients with high risk of cardiac complications due to the lack of cardiac follow-up and cardioprotective therapy. Moreover, the sustained physical exercise can worsen the cardiac condition and lead to cardiac failure⁹⁶.

A pseudo-metabolic presentation is commonly reported in BMD patients reporting muscle cramps, effort intolerance, recurrent myoglobinuria and rhabdomyolysis episodes.

FEMALE CARRIERS

The BMD female carriers can have clinical manifestation in 5 to 22% of cases^{55,84,97,98}. The diagnosis in young girls is usually secondary to an abnormal blood test with high liver enzymes and CK. The skeletal muscle symptoms can vary from cramps and effort intolerance to muscle weakness of variable degree. The main risk is the cardiac complications due to a sustained physical exercise and lack of cardiac follow-up and preventive treatment. The cardiac injury is similar to the classic BMD presentation with a progressive systolic failure complicated with a dilatative cardiomyopathy and ultimately with a cardiac failure.

DIAGNOSIS

In the case of the absence of family history the diagnosis is based on the typical clinical presentation in adult male. As the clinical presentation is confirmed, the blood investigation will complete the diagnosis with the detection of the elevated CK blood level, eventually a muscular biopsy is proposed in case of atypical clinical presentation in order to perform a differential diagnosis for the metabolic presentation with effort intolerance, myoglobinuria and rhabdomyolysis. The diagnosis confirmation will be genetic. In contrary, for the presentations with a positive family history and a positive clinical presentation a genetic test is proposed in first intention^{94,95}.

The diagnosis is frequently delayed in the case of infant presentation. The developmental delay or the behavior and speech disabilities can delay the examination of this boys by experimented pediatric neurologist for diagnostic confirmation. Once well oriented, the CK elevated level and in some cases, positive family history will conduct to genetic testing. The muscular biopsy remains optional.

ELECTROMYOGRAM (EMG)

There is no use of EMG investigation for diagnostic purposes except for the atypical presentations. The motor and sensor conduction study it's normal with a typical myogenic profile and absent decrement. Sometimes, if the investigation is conducted at advanced stages of the dystrophy with a large area of muscle inflammation and necrosis, the myogenic profile is abolished and there is a mix or a pseudo neurogenic profile.

MUSCLE IMAGING

The muscle imaging is optional in case of clinical research follow-up trial in specialized centers and is not necessary for the diagnostic confirmation except for atypical presentation. In the large majority of presentation, the topography of affected muscles are as follows: *medius* and *maximus gluteus*, *quadiceps*, *longus* and *magnus adductors*, *semitendineus*, *semimembraneus* and *biceps femoris*. At the upper limb the triceps lateral a long head and the *biceps brachii*. The dystrophy presentation is symmetrical and the deltoid and coraco-brachialis muscles are spare. The choice of the imaging is predominantly by Magnetic Resonance Imaging (MRI) for the topography identification and severity evolution follow-up⁹⁹⁻¹⁰¹.

MUSCULAR BIOPSY

The muscular biopsy remains important in the era of genetic testing as it provides useful information of the neuromuscular process in case of negative family history. It is usually performed in surgical conditions depending on the protocol of the reference center where it is conducted. During the biopsy a small sample of muscle is collected by ever open or needle biopsy. The advantage of the needle biopsy is the procedure length quicker than an open biopsy but unfortunately frequently the quality of the collected tissue remains poor for some specific histologic analysis and the quantity is sometimes insufficient for all the required analysis. The

majority of French reference centers for neuromuscular disorders have chosen to practice the open biopsies and the most frequent biopsy area is the deltoid of the quadriceps muscles and the biopsies weight average is around 20-30mg. The quality of the biopsy and also the degree of dystrophy of the biopsied muscle is important to be taken into consideration for the further analysis. Once, harvested, one fragment of the biopsy is snap freezed in isopentane for the diagnosis analysis and the second fragment, is placed in liquid nitrogen for -80° storage. Particular care is taken in order to store the second fragment without the interruption of the cold chain for Western Blot (WB) analysis.

THE MICROSCOPY ANALYSIS

The microscopy analysis allows the experienced pathologists to use immunohistochemistry analyses to confirm the dystrophy by the association of the necrosis process (pale fibers at hemtoxyline-eosine-safran dye), of regeneration (small fiber and basophiles) and fibrosis. There is also a fiber size variation, with small multinuclear fiber, and bigger ones. In some advanced stages there is an important adipose and fibrosis infiltrate of the muscle at endomysium and interstitial levels overcoming the muscle tissue. But also, depending on the intensity of the dystrophy the sparce difference between the fiber size along with rare regions of necrosis and central nuclei can be the only manifestation at the early timepoint of the diseases^{102,103}. Interestingly, Kaido et al 1991, described a difference in the BMD muscle pathology depending on age at the biopsy. As mentioned, in younger patients (≤ 15 years old), the pathology is manifested predominantly by the features of necrosis and regeneration, whereas, in patients >15 years old, the chronic myopathic changes are predominant with increased centralized nuclei, split fibers, fiber size variation and endomysial fibrosis¹⁰⁴. There are mainly three methods of analysis of dystrophin protein, the Archevala-Gomez, Taylor and Beckman¹⁰⁵. In the first method the muscular sections are labeled with dystrophin only, the circles are placed overs the selected areas of sarcolemma and maximum membrane intensity within the circles is calculated¹⁰⁶. In the Taylor method, sections are dual labeled with dystrophin and spectrin (a plasma membrane surface cytoskeletal protein) and the average of dystrophin intensity from all the fibers are calculated¹⁰⁷. In the Beekman et al 2014 the sections are again dual-labeled with dystrophin and spectrin and the distribution of dystrophin intensity for each fiber is calculated¹⁰⁸.

The three of them has been implemented successfully for several years and recently⁹² an expert consortium have demonstrated the excellent interrater reproducibility in two of them (the Beekman method was used only by one participating center). Despite these time proofed methods of measurement of dystrophin intensity there are some new methods currently in use like the MuscleMap. This method has the advantage of assessing the dystrophin intensity in individual fiber identified by a membrane-associated marker (i.e. laminin- α 2 or spectrin) in a duplex immunostaining assay. In addition, MuscleMap software includes a measure of the circumference of the membrane with dystrophin or utrophin expression), biomarker intensity and morphometric features (such as minimum fiber diameter). Also, the morphometric measuring feature will assess levels of intra- and inter-fiber heterogeneity, and counts the number of revertant fibers^{109,110}.

Together, these data illustrate the diversity of the current methods of dystrophin analysis in IF-stained sections measuring intensity in both positive and negative fibers, measuring mean vs. maximum intensity vs. distribution of intensity, and measuring intensity in a portion of the membrane vs. the entire membrane. Overall, continuous efforts are made in order to improve the actual image analysis techniques to overcome biases in sample selection as well as manual scoring. Many of the presented methods are currently used in order to accurately and consistently measure biomarker changes in clinical trials.

DYSTROPHIN QUANTIFICATION

The measurement of dystrophin expression in BMD/DMD muscle biopsies is an important step of diagnosis process and a challenging biochemical outcome measure for the clinical trials. The relative quantification of dystrophin protein is performed by the western blot technique (WB). The WB is a method to detect proteins by transferring (blotting) proteins separated by electrophoresis from a gel to a membrane corresponding to their molecular weight. The muscular isoform of the dystrophin protein is a large-scale protein of 427kDa. For this large-scale protein is difficult to have a standard. Thus, a standard curve is proposed to be used from the extraction of healthy control muscle. The current practice is to report the dystrophin amounts in terms of percent dystrophin found in healthy control (e.g., percentage of healthy control where the control is assumed to be 100%). In BMD patients, there are very few thorough studies of dystrophin expression. The reported dystrophin expression values in patients with BMD vary dramatically due to multiple factors as the natural variability of dystrophin levels

across patients. Moreover, the difficulties in dystrophin quantification are enhanced by the presence of pre-existing revertant fibers with residual trace dystrophin and the large size of the protein and its low expression in some particular, out of frame, genetic mutation of *DMD* gene and ultimately by the method of detection^{57,92,105,111}. As demonstrated in a previous work of our team, the dystrophin quantification by WB in BMD patients is around 60-70% compared to healthy subjects⁷⁴. Actually, there is a number of challenging clinical trials with dystrophin correction and replacement therapies in DMD. Thus, the methodology of WB became very important as it is used as secondary outcome measure in a large number of clinical trials in DMD.

In addition to the described methods, there are alternative methods such as mass spectrometry and ELISA for the detection of the linear increments of dystrophin from very small amounts of biological samples.

We reported here the detailed clinical phenotype in Becker muscular dystrophy and highlighted the highly heterogeneous feature of BMD. However, literature data is based on detailed clinical reports but larger cohorts with similar mutation pattern are still missing. Indeed, phenotype and genotype variability were broadly reported between mutations sharing similar domains of dystrophin protein. Precise genotyping highlighted that different *DMD* gene deletions can affect the properties of the resulting dystrophin protein as for example the loss of functionally critical N- or C- terminal domains and may result in DMD-like phenotypes⁸⁷. The current advancement in genetic screening and diagnostics has the potential to complete the missing pieces and diagnostic caveats in DMD and BMD and help establish a long term clinical prognosis and therapy choice.

DYSTROPHY PATHO-MECHANISM

Generalities

The high clinical heterogeneity and genetic complexity reveal a multifactorial pathomechanism in DMD/BMD (Figure 9).

The elevations in intracellular Ca^{2+} level by “leaking channel”, increased membrane permeability, increased levels of reactive oxygen species and modifications in the expression and function of nitric oxide synthase, NADPH oxidase and caveolins proteins are considered to take part to the dystrophic process³⁵.

In order to address causal gene relationship into the cellular and molecular mechanisms animal models have been used despite some pathological differences to humans. The mice model is frequently used in preclinical studies for proof-of-principle, dosage and efficacy studies. Larger animal models as golden retriever dog model (GRMD) are also of major interest in pathomechanism and preclinical development. The GRMD model has a naturally occurring mutation at intron 6 in *DMD* gene¹¹⁰. Also, different type of mutations has been described in canine model. In mice, there is a naturally occurring dystrophic mouse strain displaying a point mutation, changing the amino acid coding for glutamine to stop codon in exon 23 of *DMD* gene (*mdx*, X-chromosomal linked muscular dystrophy). The *mdx* genotype display a mild phenotype and a near-normal life expectancy. In addition to the spontaneous mutations resulting in a muscular dystrophy phenotype, many mouse models have been genetically engineered^{110,112}. The dystrophy pathomechanism was largely described in these animal models.

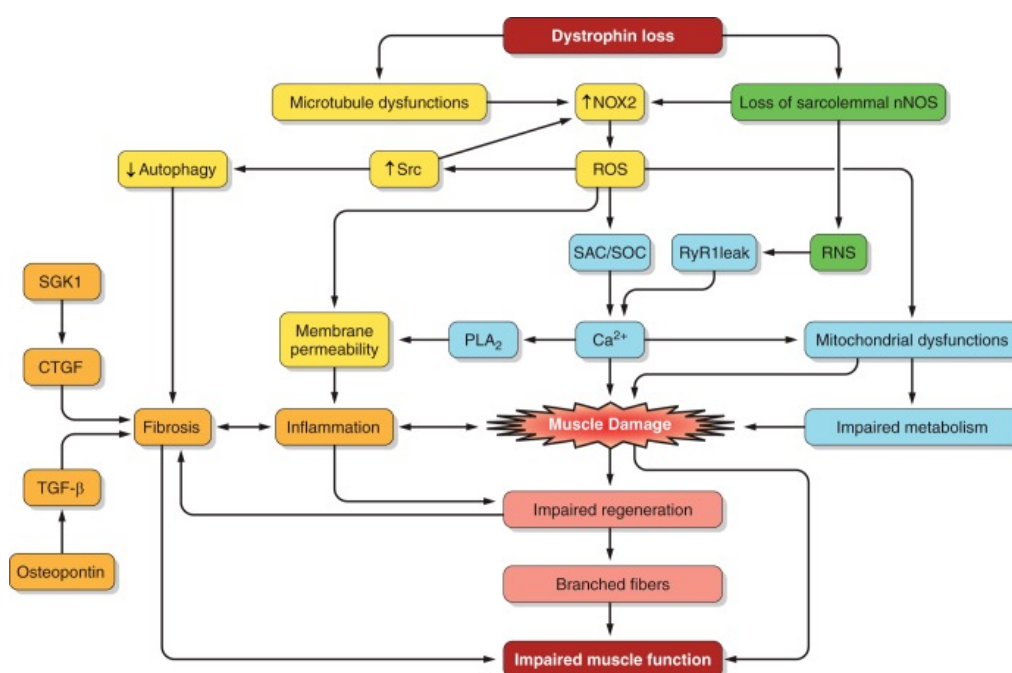


Figure 9 Scheme presentation of dystrophic pathomechanism process.

Adapted from Allen 2016

CALCIUM FLUX

The first studies in the mechanisms involved in the pathogenic process in DMD muscle have revealed, by electronic microscopy (EM) evidence, focal disruptions in the skeletal muscle cell membrane and elevations in intracellular Ca^{2+} ³⁵(Allen *et al.*, 2016). Elevated intracellular Ca^{2+} concentration in DMD muscles suggested its role in the pathologic mechanism^{113,113}.

The hypothesis of a membrane damage caused during contraction was demonstrated in *mdx* mice model^{35,114}. In addition, there is a demonstrated correlation between the decreased Ca^{2+} release rate and sustained muscle contraction¹¹⁵. Recently, new studies showed that Ca^{2+} pathway is involved in fatigue, a constant symptom in BMD/DMD patients. Literature data underline an important link between fatigue muscle, Ca^{2+} and sarcoplasmic reticulum (SR)¹¹⁶⁻¹¹⁸. These observations are based on the study of fatigue muscle and the measurement of vesicle

Ca²⁺-ATPase activity, as well as SR vesicle uptake and release rates. Moreover, the dystrophin loss combined with Ca²⁺ influx and muscle contraction results in muscle damage in *mdx* mice. Also, evidence of myonecrosis and regeneration was found in muscle damage during unlimited activity versus in limb muscles kept at fixed length^{35,114,119,120}.

NITRIC OXIDE (NO)

Dystrophin protein has multiple protein partners and among these multiple connections an important role resides for the nitric oxide synthase (NOS) protein³⁷. There are three distinct genes coding of nitric oxide synthase (NOS) protein: *NOS1* (nNOS), *NOS2* (inducible NOS, iNOS) *NOS3* (endothelial NOS, eNOS) and the nNOS isophorm is the most predominant from the four known isoforms. The nNOS protein with a specific insert (μ) is muscle specific and has complex functions. In skeletal muscle nNOS is localized at the sarcolemma and cytoplasm. Associated with DAPC, nNOS acts as a signalling protein⁷⁰. The produced NO may regulate the adaptation to exercise in skeletal muscle by the modulation of excitation-contraction coupling, glucose uptake, regulation of vascular perfusion during contraction and calcium mobilization in healthy muscle. Indeed, in healthy skeletal muscle nNOS μ allows the release of NO into the muscle vasculature during contraction resulting into a vessels dilatation. The nNOS μ mislocalization from membrane will result with focal ischemia and necrosis.

Interestingly, a recent study from our group reported that cytosolic mislocalization of nNOS μ correlated with hypernitrosylation of the SR ryanodine receptor type-1 (RYR1) and release of calstabin-1 in skeletal muscle biopsies from BMDdel45-55 patients⁷⁴.

These listed functions and also the role of nNOS and NO in the increase of reactive oxygen (ROS) and nitrogen species (RNS) highlight its involvement in DMD and BMD pathology^{35,84}. As expected, in DMD patients reduced nNOS results in ischemia and impaired vasodilatation but also increase in the ROS producing NADPH oxidase NOX2^{84,92,121}. Recent studies identified that nNOS requires α -syntrophin for the DAPC association. The 16-17 repeat of the dystrophin rod domain is necessary for the nNOS association^{70,121}. In *mdx* model the AAV-mediated dystrophin constructs including the spectrin-like repeats 16-17 in the rod domain reported a beneficial role for the nNOS restoration. The importance of the nNOS restoration in DMD patients will hopefully be revealed by the comparison of the AAV-mediated

microdystrophin construct among the three microdystrophin constructs that are currently tested in clinical trials (*cf.* section 6). Interestingly the loss of nNOS from sarcolemma is not only specific to muscular dystrophies but also in inflammatory myositis, in amyotrophic lateral sclerosis and myasthenia gravis suggesting that nNOS participates in the neuromuscular junction function¹²²⁻¹²⁴. In these models, with defective transmission at the neuromuscular junction, the associated partners as α -syntrophin and dystrophin are present. As mentioned the nNOS protein is localized also in cytoplasm and is associated with microtubules. The microtubule-associated proteins (MAP) have been involved in the signal transduction by the modulation of microtubules and by the interaction with nNOS and the α -syntrophin. Interestingly, the microtubules interaction is important in the maintenance of the lattice structure of the healthy muscle by the interaction with dystrophin. Consequently, microtubules dysfunctions are supposed to influence calcium and ROS signalling¹²⁵. The debate is still open on the role of nNOS in dystrophic process as it remains unclear if abnormalities in nNOS are at the origin or a consequence in the pathogenic process.

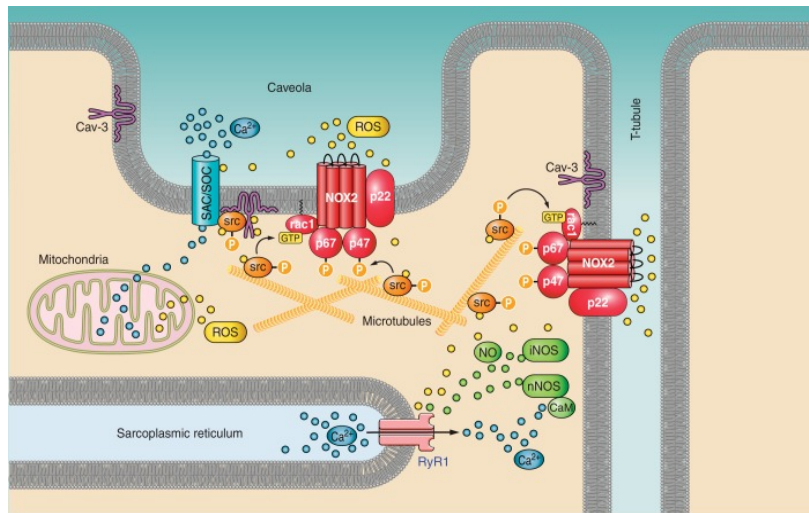


Figure 10 ROS mechanism in dystrophic muscle.

Adapted from Allen et al. 2016.

REACTIVE OXYGEN SPECIES (ROS)

ROS are chemical species containing oxygen molecules. In a biological context, ROS are the result of the normal metabolism of oxygen, they chemically react with lipids, DNA, and proteins to modulate their structure and function. Based on their function ROS are important actors in cell signaling and homeostasis¹²⁶. The disequilibrium in ROS production and/or reduced scavenge function will result in a pathological condition characterized by cellular damage and defined as an oxidative stress¹²⁷.

As described above, in skeletal muscle multiple pathophysiological conditions such as hypoxia, metabolic disorders and aging can result in oxidative stress as depicted in Figure 10. Consequently, this will lead to impairment of muscle function, reduced regenerative capacity, atrophy, and finally muscle weakness³⁵. On the other part, ROS are recognized to participate to many physiological functions as muscle regeneration, mitochondrial function and muscle hypertrophy¹²⁸. Based on their versatile functions, antioxidants were tested in several clinical trials in DMD. Despite the lack of improvement in muscle strength, which was the primary outcome, some improvements were reported overall¹²⁹.

THERAPIES

Generalities

DMD is a lethal genetic disorder but several new therapies are currently available to eligible DMD patients with specific mutations. As reported here, the multifactorial character of dystrophin injury patho-mechanism reveals the complexity of the therapy development.

The standard of care includes the use of corticosteroids and prevent or assist the cardiac, respiratory and orthopedic secondary complications^{83,86,88}. In the last decade the corticosteroids offered multiple benefits in DMD patients by stabilizing muscle strength and function, prolonging independent ambulation and delaying the progression of scoliosis and cardiomyopathy⁹⁵.

In addition, several different therapy approaches are evolving with the aim to transform the severe DMD in the BMD form of the disease. These therapies assume that BMD is a mild disease. However, this is not completely true as BMD patients have a highly heterogeneous clinical presentation. Currently there is no molecular basis explanation of the clinical heterogeneity of the BMD. Despite a less severe presentation than DMD and a preserved life span, BMD patients have a slowly progressive clinical presentation with skeletal muscle deficit in a large number of BMD patients. Currently there is one clinical trial for BMD patients and this limited number is probably reflecting the difficulties in development of clinical trials in this highly clinically heterogeneous population (Table1).

PREMATURE STOP CODON MUTATIONS

The premature stop codon mutation of DMD gene arise in approximatively 10%-15% DMD patients. As indicated by the name, the nucleotide triplets within mRNA release prematurely the signal of the termination of the protein translation. In result, the ribosomal subunits dissociate and the protein is shortened and usually lacking critical amino-acid parts. TranslarnaTM (ataluren) stop-codon read-through therapy binds to the ribosomal subunits and prevent the premature stop codon recognition (NCT02369731). This is the first EMA/FDA

condition approval therapy and the latest safety and effectiveness comparison results from the STRIDE Registry and CINRG DMD Natural History Study were reported recently. According to the authors, children treated with Translarna in a real-world setting as part of STRIDE registry were able to walk independently for an additional 3.5 years compared with a propensity score matched cohort in the CINRG natural history study with a median age at loss of ambulation of 14.5 years and 11 years and free of significant adverse effects. Final data from the STRIDE registry are expected in 2025⁸⁸.

Therapy	current status
Stop-codon read-through	
Ataluren / Translarna®	AMM registry STRIDE
Exon skipping therapy	
AVI-4658 / Exondys51® / eteplirsén (skipping exon 51)	Phase III
SRP 5051 (skipping exon 51)	Phase II
NS-065/NCNP-01 / viltolarsén/ Viltepso® (skipping exon 53)	Phase III
SRP 4045 / casimersén (skipping exon 45)	Phase III
NS-089/NCNP-02 (skipping exon 44)	Phase I/II
DS-5141b (skipping exon 45)	Phase I/II
WVE-21020	on hold
scAAV9.U7.ACCA (skipping exon 2)	Phase I/II
Gene therapy	
AAVrh74.MCKMicro-Dystrophin (SRP-9001)	Phase II
PF-06939926	Phase II
SGT-001	Phase I/II
AAV-micro-dystrophin (<i>Généthon</i>)	in progress
Cell therapy	
Myoblasts transplantation	Phase I/II
CAP-1002 – cardiac stem cells	Phase II
Bone marrow stem cells	Phase I/II
Utrophine upregulation	
GALGT2 - rAAVrh74.MCK.GALGT2 gene therapy	Phase I/II
Myostatin inhibitors	
Givinostat	Phase III/DMB
Givinostat	Phase II/BMD
RG6202 / R07239361 / Adnectine	on hold
Domagrozumab	on hold
Fibrosis reduction	
Tamoxifène	Phase III
Pamrevlumab	Phase II
Inflammation inhibitors	
Prednisone/prednisolone	standard of care therapy
Prednisone/prednisolone	Phase II
Deflazacort / Emflaza	Phase IV FDA approved
Vamorolone (VBP15)	Phase I/II
Edasalonexent (CAT-1004)	Phase III
Spirolactone	Phase I
Canakinumab (ILARIS)	Phase I/II
TAS-205	Phase II
ATL1102	Phase II
Mitochondria therapy	
Sarconeos (BIO 101)	in progress
ASP0367 (MA-2011)	Phase I
RedOX	
Idebenone	Phase III
NO-synthetase stimulation	
Citrulline et Metformine	Phase III
Cardioprotective therapy	
Néivolol	Phase III
Bisoprolol	Phase II/III
Spirolactone	Phase III
Riméporide	-
Ifetroban	Phase II
Epicatchine	Phase I

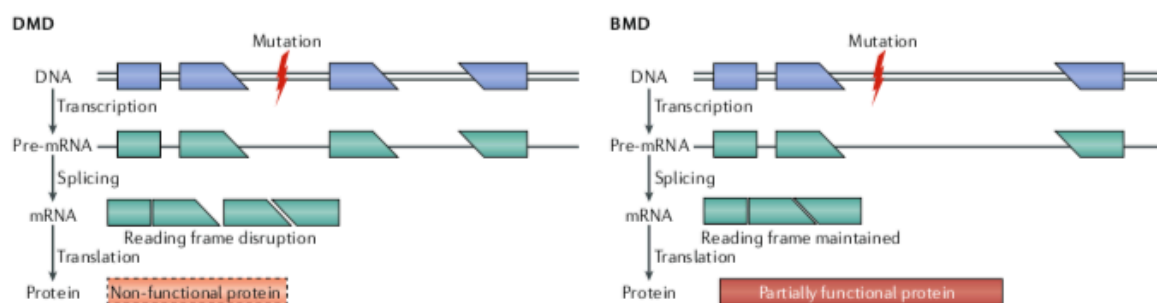
Table 1 DMD/BMD therapies adapted from AFM Téléthon

EXON SKIPPING

Exon skipping and exon inclusion drugs as Exondys 51 (Eteplirsen) and Spinraza (Nusinersen) are the first FDA/EMA approved therapies in DMD and spinal muscular atrophy (SMA).

This strategy was developed in regard to the BMD in-frame mutations that result in a milder phenotype and present, partially truncated but functional dystrophin protein. In DMD, antisense oligonucleotides sequences (AON) are currently use to correct specific dystrophin gene mutations by exon skipping. This strategy aims to force the skip of specific exon(s) during the splicing process of preRNA maturation. Indeed, AONs are single-stranded synthetic nucleic acid chains designed to mask specific sequences used by the splicing machinery which can therefore no longer recognize the exon which is skipped with its adjacent introns. Exon skipping was first induced by targeting exon-internal sites, but in order to improve the precise recognition by the splicing machinery, the strategies are currently targeting the exonic splicing enhancer (ESE) motifs^{130,131}. The restoration of the reading frame by the induction of the specific exon or multi-exons skipping results in the partial production of an internally truncated protein as depicted if Figure 11.

In phosphorodiamidate morpholino backbone (PMO), a third generation of AOs, a non-ribose-based modification prevents degradation by nucleases, but the non-ionic structure of these compounds from nuclease activity but their non-ionic structure reduces their nuclear uptake¹³². In the latest generation of AOs, short cell-penetrating peptides have been conjugated to PMO (PPMOs), to increase their ability to penetrate into cells^{133,134}. PPMOs, like all the other AOs produced to date, have the enormous advantage of not being immunogenic, but they must be administered regularly to sustain therapeutic benefits in skeletal muscles and they have been reported to have only weak effects on dystrophin restoration in the heart¹³⁵.



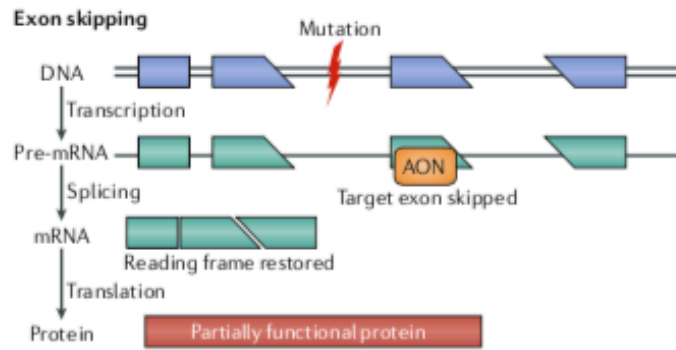


Figure 11 Exon skipping scheme illustration in DMD gene.

Exons are depicted in blue boxes, resulting protein in red. AON=antisens oligonucleotides

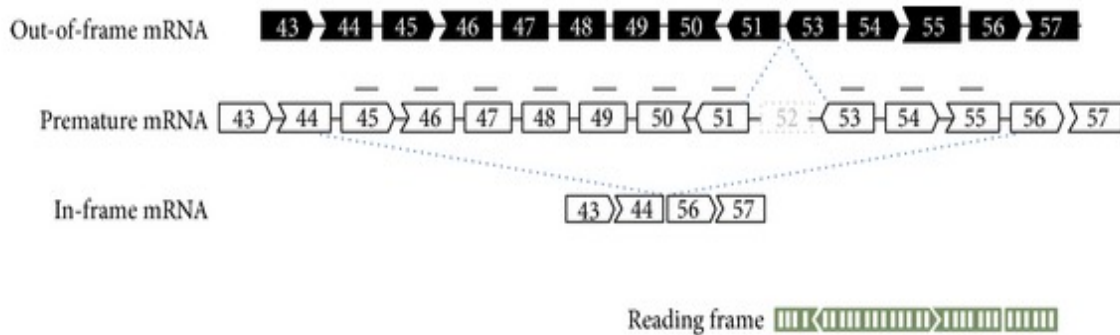


Figure 12 MES 45-55 scheme illustration.

Black exons illustrate the mRNA with a out-of-frame mutation. White boxes illustrate the premature mRNA and the corrected open reading frame. In green scheme depiction of the restored reading frame.

The first 2'-O-methyl-phosphorothioate oligoribonucleotide (PRO051) designed to skip exon 51 in dystrophin pre-mRNA did not show statistically significant improvement in treated patients for the six minutes walking test (6MWT) at 49 weeks of treatment. The phosphoramidate morpholino oligomer (PMO) (eteplirsen) designed for specific exon 51 skipping met the primary end point in phase III clinical trial⁷⁷. Despite an increasing number of FDA/EMA approved therapies (exon 51, 53, 45 skipping therapies in ongoing clinical trials)

the number of eligible patients to this specific mutation treatment remains limited (NCT03992430, NCT03218995, NCT02310906, exon 45 (NCT02500381, NCT02530905, NCT03532542, NCT04179409, exon skipping 51 (NCT03375255, NCT03675126, NCT04004065) exon skipping 53 (NCT03167255, NCT04060199) exon skipping 44 (NCT04129294), exon skipping 45 (NCT02667483),

The main limitation to the AON therapies is the specific mutation indication and the uncertain function and stability of the resulting corrected dystrophin. One of the alternatives is the multi-exon skipping strategy. Indeed, there are two main mutational hot-spots in *DMD* gene. First one located between exon 2 and 20^{62,67,136} and the second one is the largest one, involving exon 45 to 53¹¹¹. The spontaneous deletion of the exons 45-55 encouraged the development of multiexon skipping despite the initial technical caveats. Indeed, the large number of exons required the design of antisense oligo morpholino mixtures in order to minimize self- and heteroduplex formations. Aoki et al. demonstrated the feasibility of skipping exons 45-55 in H1K-*mdx52* myotubes and in *mdx52* mice by systemic delivery depicted in Figure 12^{53,137}. The systemic in vivo injection of 10 AONs induced dystrophin expression at the sub-sarcolemma in skeletal muscles throughout the body, up to 15 % of wild-type dystrophin protein levels⁶⁷. Moreover, dystrophin expression was accompanied by the amelioration of the dystrophic changes in skeletal muscle and diaphragm muscles. However, the dystrophin restoration in the heart did not reach similar level as in skeletal muscle with only 2 % of restoration despite the PMO optimization (PMO containing cell-penetrating moiety of octaguanidine dendrimer).

DYSTROPHIN GENE REPLACEMENT

Since the beginning of the development of genetic therapies oriented to the *DMD* gene replacement the main challenge was to find a construct that could incorporate the large *DMD* gene size. The viral adeno associated vectors (AAV) have limited size of the viral capsid and several attempts to address this limitation resulted into selective exclusion of dystrophin gene regions. In result, several mini- and micro-dystrophin constructs were tested over the years. Although, several micro-dystrophins constructs were described, there are only three different micro-dystrophins currently in clinical trials^{138,139}.

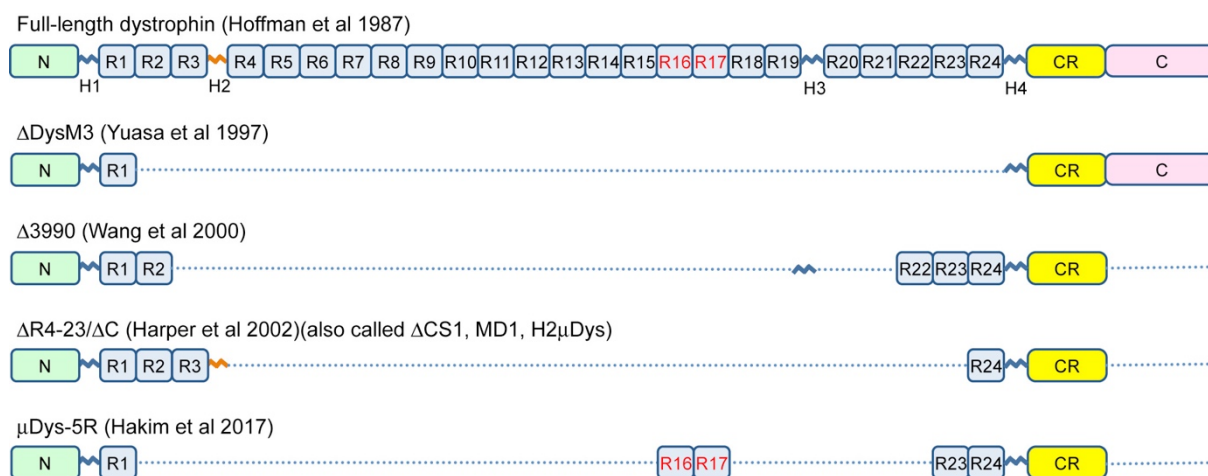


Figure 13 Micro-dystrophin constructions models in actual clinical trials (Adapted from Duan, D. 2018).

The constructions of microdystrophin have five-repeat microgene (Δ 3990), a four-repeat microgene (Δ R4-23/ Δ C) and a different five-repeat microgene (μ Dys5R) (Sarepta-NCT03375164, NCT03769116, Pfizer-NCT03362502, NCT04281485, Solid-NCT03368742)

Despite the differences in the rod domain repeats selection, all constructs have N-terminal, Cysteine-rich domains and spectrin-like repeats 1 and 24, hinges 1 and 4 and the absence of the C-terminal domain. The R16/17 nNOS-binding site is conserved only in μ Dys5R. The choice of different rod domain sites will allow the comparison of the role and the utility of hinge 2 and R16/17 as previously demonstrated in literature data^{42,140}.

Despite the currently ongoing microdystrophin trials, recently preliminary results were reported via press release and congress communications.

One of the clinical trials is currently on hold (Solid) but in the remaining two distinct microdystrophin trials encouraging results have appeared.

The rAAVrh74.MCK.micro-dystrophin (SRP-9001) results in 4 patients at 12 months indicate the absence of serious adverse events (SAE) and a high mean of dystrophin positive fibers (81%) and in 96% sarcolemmal localization of dystrophin protein. Expression was associated with an increase in β -sarcoglycan expression promoting the restoration of DAPC. Motor

function (increased ambulation, increase in muscle strength) was improved in all 4 patients and a clinically meaningful improvement on NSAA at Day 90, with a mean change from baseline (BL) to Year 2 of +7.0. The long-term efficacy will confirm these encouraging results.

The PF-06939926 (Pfizer) is an investigational, recombinant adeno-associated virus serotype 9 (AAV9) capsid carrying a shortened version of the human dystrophin gene (mini-dystrophin) under the control of a human muscle-specific promoter.

Recently Pfizer press release reported that preliminary data from 9 ambulatory boys with DMD was recently presented at the American Society of Gene & Cell Therapy (ASGCT) Annual Meeting. Nine ambulatory patients aged 6 to 12 (mean age: 8 years) received intravenous administration of PF-06939926 and was well-tolerated during the infusion period. Unfortunately, three SAE occurred but fully resolved within 2 weeks due to a probable complement activation for 2 patients. Of the 3 patients in the low dose cohort, the mean percent positive fibers were 28.5% at 2 months and 21.2% at 12 months. Of the 6 patients in the high dose cohort, the mean percent positive fibers at 2 months was 48.4%. For the 3 patients in the high dose cohort for whom 12-month data are available, the mean percent positive fibers were 50.6% at 12 months.

BL NSAA scores after one year, compared with the scores in an independent, external control group derived from recent prior clinical trials involving boys with DMD, who were matched specifically by age, weight and function (i.e. median loss of 4 points in NSAA total score for external placebo group [N=61] vs. improvement of 3.5 points in the Phase 1b patients [N=6], $p = 0.003$). Further results are necessary in order to confirm long-term efficacy.

CRISPR-CAS9 MES 45-55

Clustered regularly interspaced short palindromic repeats (CRISPR-Cas9) are RNA-guided DNA endonuclease system that allows the specific sequence targeting in the genome. The Nobel prize in Chemistry 2020 was awarded to Emmanuelle Charpentier et Jennifer Doudna for their contribution in the development of that gene editing technology. In contrast to OANs that requires multiple systemic delivery the CRISPR-Cas9 unique technology has potential for permanent exon skipping which can restore the disrupted *DMD* reading frame. Recently CRISPR-Cas9 technologies were delivered by viral tissue specific vectors in order to correct the *DMD* mutation in target tissues. This technique was designed to correct the *DMD* mutations especially in relatively inaccessible area such as the heart.

CRISPR-Cas9 system will target a precise specific sequence, the gRNA will induce a DSB (Double Strand Break) in any selected DNA sequence upon the administration of Cas9 enzyme. The designed sequence will be localized near a PAM site recognized by the Cas9 enzyme and in the most frequent cases the DSB is repaired by non-homologous end joining (NHEJ) mechanisms resulting in random insertions and/or deletions at the site of cleavage.

There are three main strategies as repair mechanisms. The single gRNA in order to target cleavage at/or near a premature codon stop in a *DMD* exon, indel formation by NHEJ reframing will restore the reading frame. The second mechanism will sgRNA to target cleavage at or near splicing sequences in *DMD* exons/introns and NHEJ will allow for classical exon skipping. The third one will use two gRNAs to target cleavage in separate exons or introns, so deletion of more exons can be achieved in order to perform single ES or MES (Kenji Lim 2018).

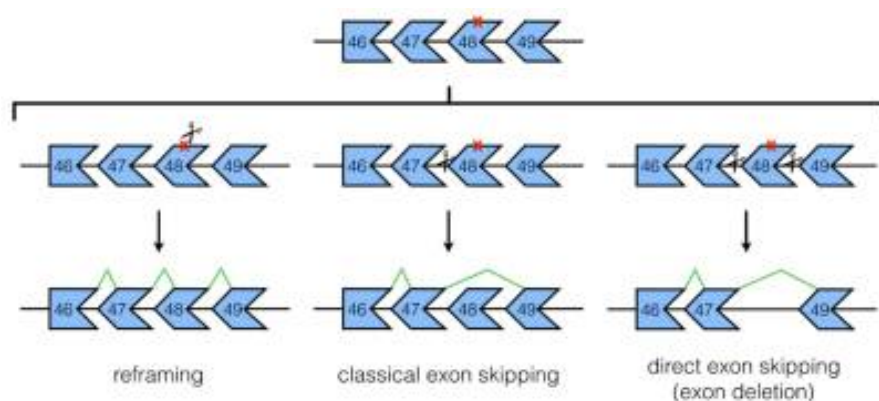


Figure 14 CRISPR-Cas9 system repair mechanism.

Adapted from Lim et al 2018 doi: [10.3390/jpm8040038](https://doi.org/10.3390/jpm8040038)

In vitro, first single exon skipping by CRISPR-Cas9 has been successfully demonstrated in iPSCs from DMD patient harboring an exon 44 deletion resulting in restoration of dystrophin expression^{141,142}. Based on these encouraging in vitro data and after the mouse models^{143,144} followed by the canine CRISPR-Cas9 study in deltaR50-MD dog model of DMD¹⁴⁵. Dystrophin restoration was achieved up to 3-90% of normal altogether with improved muscle histology. These large-animal results confirm that dystrophin restoration including in heart muscle (92%) is achieved by gene editing approaches. More studies are needed in order to

complete these initial encouraging reports with function data and immunogenic responses for the in human systemic treatment.

In conclusion, we reported here a synthesis of the main therapy development strategies but more therapies are currently in preclinical development with high potential of combined or supportive DMD therapies. Indeed, compared to the limited therapy choice for DMD patients in the recent past, currently, several exon skipping drugs are available for eligible DMD patients. Despite that, there is a growing number of negative results of phase II and III clinical trials that highlight the complexity of the dystrophic process and the need of better understanding of the pathomechanism of this complex disorder. Indeed, biomarker and personalized screening are currently in development in order to improve the design and conduct of preclinical and clinical studies in DMD. Importantly, despite a BMD milder phenotype, recent, detailed clinical reports identified that even the in-frame mutation patients can display severe muscular deficit and therefor the questions of their eligibility to current therapies should be considered.

BIOMARKERS

Biomarker characteristics is the objectively measured and evaluated indicator of normal, pathogenic biological processes or pharmacological responses to a therapeutic intervention. There are three main classes of biomarkers in BMD/DMD:

- Diagnostic biomarkers
- Prognostic biomarkers
- Therapeutic monitoring biomarkers

Respectively the biomarkers can belong to either one class or to all three main classes mentioned above¹⁴⁶.

DIAGNOSTIC BIOMARKERS

The most common used diagnostic biomarker to date in BMD/DMD is the creatine kinase (CK), a muscle enzyme that it is used as a diagnostic marker of the muscle damage. CK is elevated in the bloodstream and reflects the muscle damage but do not correlated with the disease clinical severity. Also, the CK is not specific to BMD/DMD or event to the dystrophic process because it is reflecting the muscular damage in healthy subject in case of a sustained physical effort or regeneration. However, this muscular marker remains useful in common clinical practice and helps the clinicians in the preliminary differential diagnosis elaboration.

Prognostic biomarker research is primarily oriented on genetic modifiers. Since the last decade, there is an important increase in available data on new gene modifiers in DMD disease due to the continuous development of high-throughput technologies. The DMD most known and described disease modifier is the *DMD* gene itself since its presence or absence is correlated to the clinical phenotype in the majority of cases.

PROGNOSTIC BIOMARKERS

Also, the development of the new high-quality sequencing technologies as NGS and WGS recently revealed a variety of SNP (single nucleotide polymorphism) as disease modifiers in DMD. Such examples are the SNP in the genes of *LTBP4* (latent TGF-beta binding protein 4) and *SPP1* (osteopontin) have been reported to influence the age of LoA (loss of ambulation) in DMD patients^{66,147}. Moreover, the disease modifiers can be used not only in clinical practice in

order to adapt the prognostic message delivered to the patients and young boy's families while diagnostic is initially made. Additionally, some of the SNP could be potentially used in clinical trials in order to better stratify the study cohorts and take into consideration the ability of patients to likely respond to a new potential drug.

THERAPEUTIC MONITORING BIOMARKERS

In the case of therapeutic monitoring biomarkers, a particular place is held by the pharmacodynamic biomarkers (PD) as dystrophin protein for example. There are increasing advances in the accuracy improvement of the technologies of dystrophin quantification. However, several caveats remain to be taken into consideration in regard of dystrophin quantification as a surrogate endpoint. First, it is not known to date if the amount of dystrophin is similar along all human skeletal muscles, also the fibrosis and fat level differ between muscle but also within the same muscle. Literature data provide estimation of the level of dystrophin restoration in order to achieve clinical relevance but there are still some discrepancies in regard with the final quantity definition. Especially since the results of the recently FDA/EMA AON approved therapies have demonstrated less level of restoration of dystrophin and yet achieved the predefined primary and end points of the phase III clinical trials. Prospective data will be necessary in order to assess dystrophin as a surrogate endpoint. And most importantly, it should be interpreted accordingly to the type of mutation, age of the disease progression and also therapy duration and presence of gene modifiers.

Recent development in regard to biomarkers identification in DMD concerns muscle specific miRNA (myomiRs) that are known to be correlated with the disease severity and clinical assessments of DMD patients indicating their importance in the monitoring of the DMD and BMD¹⁴⁸.

One of the first report concerning the bloodstream detection of miR-1, miR-133 and miR-206 (dystromiRs) highly the abundant detection in the serum of DMD/BMD patients. Also, in mdx mouse model their levels decreased when dystrophin was restored by exon skipping therapy. Based on these findings the authors proposed these new miR candidates to be applied in DMD diagnostic and also in the therapy monitoring¹⁴⁹. Since the first report, miR-206 was recently described as a predictive biomarker capable to distinguish DMD from BMD patients¹⁵⁰. Interestingly the GC treatment did not influence the levels of the miR 181a-5p, miR-30c-5p,

miR-206. However, the longitudinal data of 4 years surveillance in BMD/DMD patients did not detect any decrease in the detection of miR-30c-5p, miR-206. The main limitation to this report is the limited number of measurements over the study duration (between two to three measurements at different time points in the same patient).

Circulating biomarkers detection allowed to identify an early stage of muscle damage in DMD boys^{142,151}. The RET (proto-oncogene tyrosine-protein kinase receptor RET); GDF-11 (growth differentiation factor-11), cadherin-5 biomarkers had very low level of detection comparing to control in young DMD boys (4 yo). These findings confirm the clinical heterogeneity in DMD patients suggesting the beginning of the muscle damage in early infancy or even prenatally.

Since several AON exon 51 skipping therapies recently achieved FDA/EMA approval, the results of the serum levels of myomiRs were investigated in DMD patients enrolled in PMO (eteplirsen) clinical trial. Surprisingly, pre and post treated eteplirsen samples did not reach significant difference of the miR-1, miR-133a, miR-133b, and miR-206. The short duration of the study (12 weeks) and the high variability of the miR expression in addition to the small number of enrolled patients could explain the lack for significance. Additional data from a longitudinal study would be necessary in order to evaluate the utility of these specific myomiRs as pharmacodynamic biomarkers in DMD.

Recent study on miR-379 serum-level underlined the elevated detection in plasma of DMD patients in comparison to age-matched controls and the reduction of miR-379 by GC treatment¹⁴⁸. These data were completed by the identification in myoblasts of the fine tuning between the miR-370 target EIF4G2 and DAPIT at mitochondrial level.

Bloodstream analysis of the protein biomarkers revealed different groups of proteins involved in the patho-biochemical pathways involved in dystrophic process as inflammation, fibrosis and muscle degeneration /regeneration along with muscle fiber leakage. Interestingly, the detected proteins involved in proliferation and differentiation diminished with age in DMD patients while increasing in healthy controls. These data suggest a disturbance of the skeletal muscle development, more detailed data would be necessary in order to decipher their involvement during regeneration.

LONG NON-CODING RNA (LNCRNA)

Generalities

In 1978 Frederick Sanger developed a sequencing technique that generated the first ever full genomic sequence of a viral genome the bacteriophage ϕ X174^{152,153}. This discovery inspired the worldwide sequencing project, the Human Genome Project (HGP). Then in 1999 followed the sequence of the human chromosome 22 and finally using clone by clone methodology the first draft of the complete human genome in 2001. The HGP project revealed that only 1.2 % of human genome represent protein coding (PC) exons, and the 24 and 74% were intronic and intergenic non-coding DNA. Moreover, most of the genome is transcribed whether it encodes proteins or not^{154,155}. Further literature data highlighted that the genome is transcribed in many non-coding RNA (ncRNA). RNA was extensively studied since the discovery of DNA and became a pillar of a central dogma of molecular biology. First, in the late 1950's was identified that RNA is a DNA-like molecule synthesized from DNA. Then the *central dogma of molecular biology* was stipulated as the transcription of DNA gene results into RNA in the nucleus and is followed by the protein synthesis in the cytoplasm. This discovery placed RNA into the center of molecular biology. The RNA adepts suggest that prebiotic life revolved around RNA instead of DNA and proteins and give rise to the RNA World Hypothesis.

First lncRNA as *H19* and *Xist* and others were characterized but their functionality remained controversial¹⁵⁶. In the last decade with the development of high-throughput sequencing technologies led to an explosion of newly identified lncRNAs. Despite the growing number of evidences many challenges in lncRNA function and mechanism of action remain to be addressed in particular their accurate annotation and clinical relevance.

LONG NON-CODING RNA BIOGENESIS AND MECHANISM OF REGULATION

Functional relevance of lncRNA raised the question of the origin, conservation but also diversification of lncRNA along the species. The non-coding genes arise through various mechanisms, as for example, in humans a large portion of the lncRNA (40-45%) are generated by exaptation (acquisition of new functions) of the RNA derived transposable elements (TE) into non-coding genes. Additional mechanisms are represented by DNA-based or RNA-based duplications of existing genomic sequences and metamorphosis of protein coding genes (PCGs) by the loss of protein coding potential^{157,158}.

The diversification of the lncRNA may occur by the pervasive transcription of the genome and generate lncRNA genes with novel functions. The unique phenotypic traits of human, are thought to be originating from the PCG and non-coding genes of *de novo* origin. At the date, the testis and cerebral cortex are identified as the most enriched tissues for the expression of PCGs and non-coding genes of *de novo* origin¹⁵⁹.

Recently new data led to revise the definition of the coding potential of lncRNA. One of the lncRNA function in the cytosols is the association with the mono- or polyribosomal complexes as a lncRNA decay. In contrary, some lncRNA include short Open Reading Frames (sORFs) and undergo translation like in the case of *DWORF*, a muscle-specific lncRNA that encodes a functional peptide of 23 amino acids¹⁶⁰. Further analysis and proteomic studies will be necessary to expand the actual knowledge of the function and “coding” definition of lncRNA and probably introduce a possible bifunctional role of lncRNA.

The expression patterns of lncRNAs is highly dependent on different species and the kind of lncRNA. For example, in mouse, the studies of the half-life ($t_{1/2}$) range from 30 min to 48 hours similar to mRNA. Inter-class lncRNA stability studies revealed that the intronic or promoter-associated lncRNA are less stable than intergenic, antisense and 3'UTR associated lncRNAs¹⁶¹. In contrary, in human HeLa cells similar studies highlighted the higher stability of antisens lncRNAs versus mRNA. The most stable lncRNAs are the circular RNAs with the median $t_{1/2}$ of 18-27h¹⁶².

The expression of the majority of lncRNA has a specific spatio-temporal, lineage, tissue and cell specific expression demonstrated by a number of transcriptome profiling studies. In some rare cases like lncRNA are ubiquitously expressed and in a highly abundant quantity as for *MALTI*, *TUG1*. In contrary, the tissue, cell specific expression of lncRNA trend to have a low level of expression^{163,164}.

DEFINITION, STRUCTURE AND CLASSIFICATION OF LNCRNA

lncRNAs are a class of RNA transcripts characterized by a length > 200 nucleotides (nt) and have no protein coding potential. In contrast to the micro RNA < 20 nt the lncRNA are poorly expressed and have a tissue and time specific developmental patterns^{165,166}.

lncRNA are highly versatile and dynamic molecules with secondary and tertiary structures. Due to their large size, it has been stipulated that lncRNA are poorly conserved¹⁶⁷. Growing number of studies reveal that the specific flexible and dynamic secondary structures define lncRNA as primary functional unit and serve as evolutionary lncRNA interspecies sequence conservation¹⁶⁸⁻¹⁷⁰. Also, a very specific lncRNA feature is the various residence nucleus specific (*NEATI*) or both cytosol and nucleus (*HOTAIR*) or exclusively in cytosol (*DANCR*)^{171,172}. The environmental changes, like in the case of stress induced lncRNA can induce a delocalization or an active trafficking from nucleus to cytosol¹⁷³. lncRNAs have been demonstrated to be involved in the processes of transcriptional activation and/or repression, chromatin remodeling and RNA splicing activation¹⁷⁴.

In recent years, due to technical advances in the deep sequencing, a multitude of lncRNA have been studied and their classification was based in regard to their length, transcript properties, location in respect to PCG, regulatory elements (Table 2).

lncRNA category	Abbreviation
Classification according to lncRNA length	
long non-coding RNA	lncRNA
large non-coding RNA	
very long intergenic ncRNA	vlncRNA
macro lncRNA	
Classification according to lncRNA location in regard to PCGs	
<i>Intergenic lncRNA</i>	
long intergenic ncRNA	lincRNA
large intervening ncRNA	
long intervening ncRNA	
antisens lncRNAs	
natural antisens transcripts	
antisens lncRNA	
SINE B2 containing RNAs"	
<i>Bidirectional lncRNA</i>	LUAT, uaRNA
<i>Intronic lncRNAs</i>	TIN, ciRNA, circRNA, sis
Classification according to lncRNA function	
scaffold lncRNA	
<i>Architectural lncRNA</i>	
guide lncRNA	
Ribo-activator	
Ribo-repressor or decoy	
competing endogeneous RNA	
lncRNA precursors	
Classification according to lncRNA subcellular localization or origin	
<i>Nuclear lncRNAs</i>	
GAA repeatcontaining RNAs	GRC-RNA
Chromatin-enriched RNAs	cheRNA
Chromatin-associated RNAs	CAR
<i>Mitochondrial ncRNAs</i>	mtncRNA

Table 2 lncRNA classification according the length, function, origin and localization.

Various mechanisms of actions of lncRNA are depicted in Figure 15. The scaffolds function of lncRNAs in the assembly of ribonucleoprotein (RNP) complexes is classically implied to the complex and dynamic three-dimensional structures with high affinity for proteins. Scaffold lncRNA are often actors of epigenetic and transcriptional control of gene expression regulation by actin in *cis* or *trans* to its transcription site. Also like in the case of *HOTAIR* lncRNA there are numerous targets in nucleus in order to affect the histone modification and gene silencing

but also in the cytoplasm, *HOTAIR* facilitates ubiquitination and proteolysis of Anataxin-1 and Snurportin-1 in senescent cells¹⁷⁵. The architecture lncRNAs (arcRNAs) reveal a particular nuclear substructure enriched in repetitive sequences indicative of complex RNA folding that is essential for their scaffold function. Interestingly these lncRNA substructure can be specifically regulated by stress, during specific stages of development or in pathological conditions. The actual recognized function of arcRNAs is to sequester proteins resulting in gene expression modifications.

The function of guide lncRNA is to recruit RNP complexes to specific chromatin loci. Also, the cell-/tissue-type or the development and also the pathology stage can modify the function of the same guide lncRNA. The ribo-repressor function is displayed by the decoy lncRNA through the induction of allosteric modifications, the inhibition of catalytic activity or by blocking the binding sites¹⁷⁶. In the contrary, the ribo-activators are essential for promoting activities usually by activation of the transcription factors. The competing endogenous RNAs (ceRNAs) also the sponges by circRNAs act by competing for miRNA binding and posttranscriptional control. The circRNAs share partial sequence similarity to PCG transcripts and the balance between the mRNA, miRNA and circRNA represents the complexity of the regulation of stress response or pathological environment¹⁷⁷.

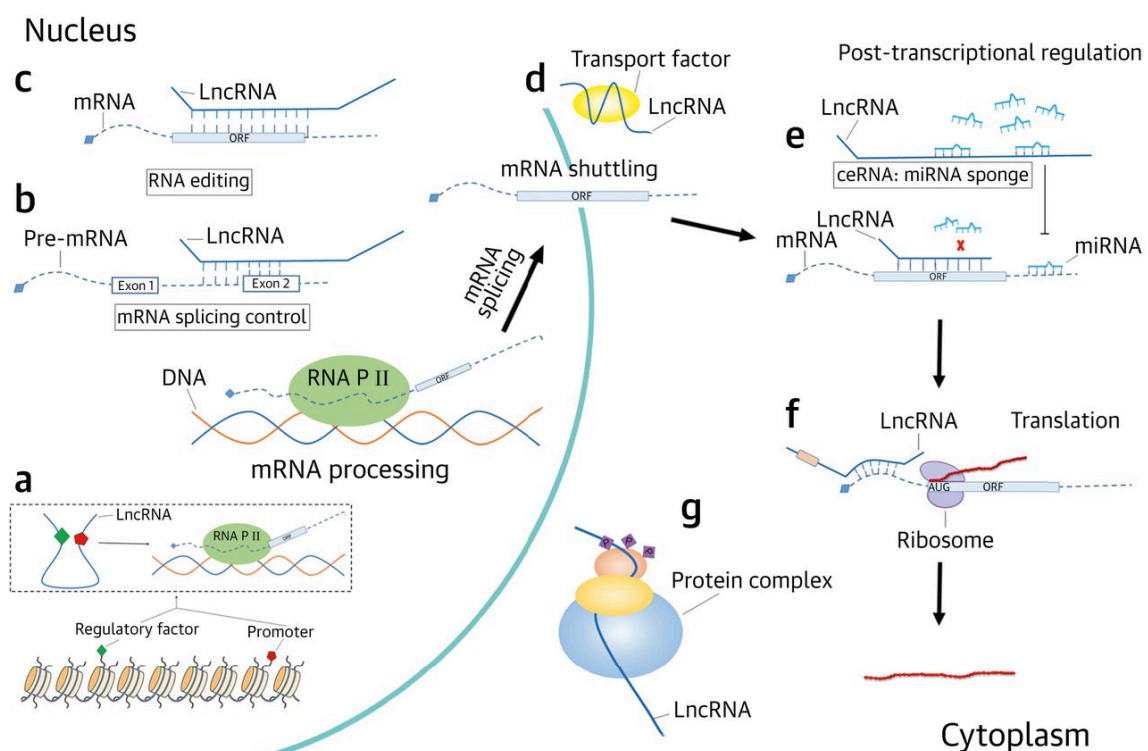


Figure 15 lncRNAs Regulatory Mechanisms.

(A) Long noncoding ribonucleic acids (LncRNAs) modulate chromatin architecture and enhancer-promoter looping at cognate genes to influence transcription. (B) LncRNAs can regulate messenger RNA (mRNA) splicing by interfering with pre-mRNA processing. (C) LncRNAs, in particular antisense lncRNAs, may direct mRNA editing through base pairing interaction. (D) By acting as decoys, lncRNAs influence the folding of complex 3-dimensional structures affecting mRNA cytoplasmic shuttling and localization. (E) LncRNAs regulate mRNA stability by acting as sponges to sequester microRNAs. (F) LncRNAs may influence protein translation through interactions with binding of translation cofactors and regulators. (G) LncRNAs may influence post-translational protein modification. ceRNA = competing endogenous RNA; miRNA = microRNA. Adapted from Zhang 2019

LNCRNA IN SKELETAL MUSCLE AND DYSTROPHIES

One of particular feature of skeletal muscle is the remarkable capacity to the regeneration due to the satellite cells. Satellite cells are quiescent stem cell population of the mature muscle tissue and are activated in case of stress or muscular damage in order to regenerate the damaged muscular tissue. The stem cells will undergo the active cell cycle division and will activate a myogenic process similar to the embryonic phase²⁰. One of many described functions of lncRNA is the myogenesis regulation.

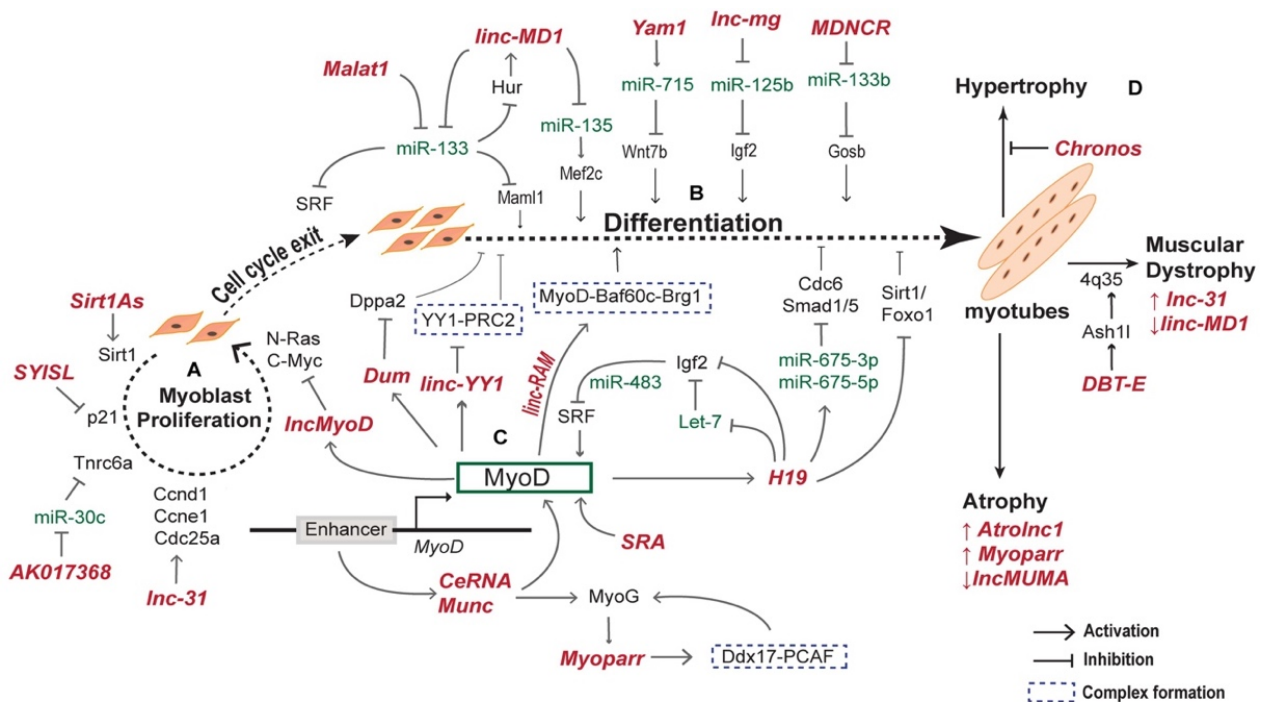


Figure 16 lncRNA involved in myogenesis and dystrophic process.

Adapted from Caretti et al., 2016

Among the first described lncRNA involved in the skeletal muscle differentiation was the SRA (steroid receptor RNA activator). Indeed, along with SRA also MyoD interact with two helicases, well known RNA metabolism enzymes (DEAD box RNA helicases as p68/ p72). Interestingly luciferase gene construction experiments in fibroblasts with muscle specific creatinine kinase enhancer demonstrated the coupled co-regulation of MyoD during differentiation by SRA and the RNA helicases as p68/ p72¹⁷⁸.

Moreover, SRA was found to have a particular feature as both, a non-coding RNA and a protein (SRAP) due to an alternative splicing. The non-coding SRA transcript is abundantly expressed in differentiating myoblasts and exerts an activation of MyoD. In contrast, the protein SRAP physically binds to its transcript homolog SRA preventing the MyoD activation and preventing the differentiation process¹⁷⁹.

Linc-MD1 were found to be abundantly expressed during differentiation in myoblasts and interestingly in newly regenerating muscle fibers. Bioinformatic studies have identified the conservation of binding sites for miR-133 and miR-135. Indeed, Linc-MD1 was reported to be associated with muscle specific miR-133 and miR-135 as a competing endogenous RNA (ceRNA). Linc-MD1 sequesters miR-133 or miR-135 as a sponge and will interrupt the binding of these miR to transcription factors required for the myogenesis as MEF2C and MAML1¹⁸⁰. This fascinating performance is enhanced by the specific regulation of the linc-MD at the cytoplasmic level. Lengini et al. demonstrated that by the binding of linc-MD to the HuR, a specific RNA binding protein. Interestingly, HuR itself is under the repressive control of miR-133 and that there is a reinforced sponging activity of Linc-MD1 acts as a feedforward positive loop¹⁸¹.

In addition to the lncRNA described in the regulation of regeneration, Bovolenta et al reported a study on the pathogenic mechanism underlying clinical manifestation in DMD female carriers. Precisely, a custom-made tiling array that explored the entire *DMD* gene and identified non-coding transcripts originating within the dystrophin locus. In this report, the sense and antisense oriented lncRNAs expression profiled mirror *DMD* gene. Precisely, the lncRNAs were localized in introns and to the nucleus. lncRNA44s, lncRNA 44s2 and lncRNA 55s are isolated from skeletal muscle and transfected in human muscle and neuronal cells. The over expression of lncRNA44s 44s2 and 55s caused a negative regulation of endogenous full-length dystrophin brain (Dp427b), muscle (Dp427m) and Purkinje (Dp427p) isoforms. Interestingly, no variation was observed with regard to the DP71 transcript which has

ubiquitous expression, suggesting the lncRNA specificity. The luciferase reporter gene on the minimal promoter region of the muscle dystrophin isoform highlighted a downregulatory role of lncRNA on full-length dystrophin. In order to confirm this specific repressive relationship lncRNA expression, function and mechanism of action should be tested in a DMD or BMD in vitro dystrophic model⁷⁹.

In conclusion, lncRNA were recently described as key players in skeletal muscle differentiation and proliferation regulation. Recent studies reported here highlighted lncRNA as regulators candidates of genotype-phenotype correlation in BMDdel45-55 patients and thus of potential importance for a detailed characterization of BMDdel45-55 population.

NEXT GENERATION SEQUENCING (NGS)

Generalities

In the last two decades the high-throughput DNA sequencing revealed a large genetic variety. Rapid decrease in costs and tremendous technical efforts allowed the detection by a single reaction of the vast majority of genetic variants. Indeed, the advent in Next Generation Sequencing (NGS) allowed the discovery of novel disease genes and variants¹⁸². Initially the high cost in genome sequencing did not allow a wide utilization of this technique in diagnostic purpose but several research project as Genomics England Clinical Interpretation Partnership (GeCIP), French Exome (FREX) in rare, life threatening and debilitating neuromuscular diseases allowed to prove their utility and efficacy.

WHOLE GENOME SEQUENCING (WGS)

Among these technologies, the WGS, has the propriety to examine single-nucleotide variants (SNVs), indels, structural variants (SVs) and copy number variants (CBVs) in the protein coding sequences and the non-coding genome. This unique feature of covering the non-coding part of the genome offer a more reliable sequence coverage with more uniformity¹⁸³.

Also, the decrease in cost and the increase in the ability to perform WGS in relative short timeframe result in a large volume of data, becoming a powerful tool in genomic research.

A fine example of opportunities and challenges of WGS in Charcot-Marie-Tooth disease is illustrated in Figure 17 and underlines the importance of deep phenotyping in diseases with highly heterogenous clinical presentation¹⁸⁴.

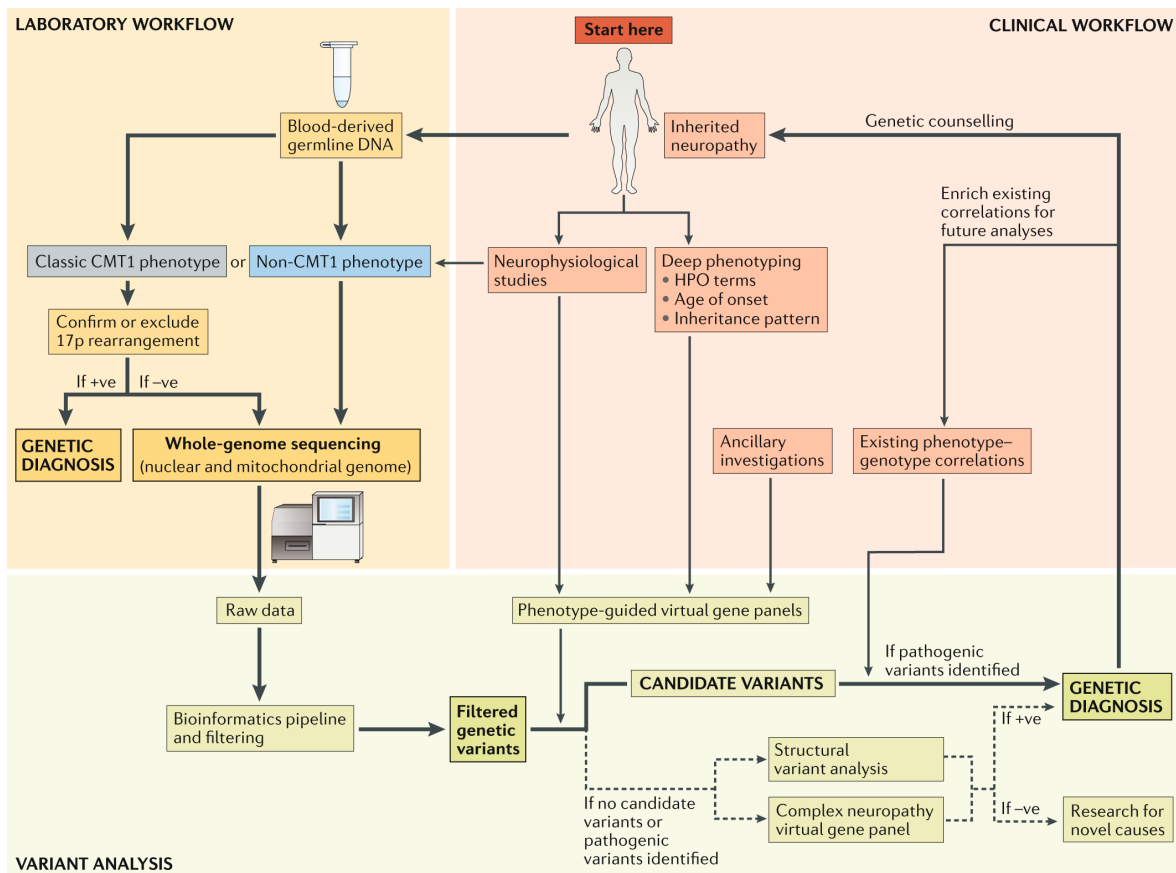


Figure 17 Charcot-Marie-Tooth disease phenotype genotype analysis using WGS.

Laboratory workflow propose to identify suitable candidates to WGS, then sequenced data of both nuclear and mitochondrial genomes are analyzed and filtered according to bioinformatic pipeline. The patient characterization is performed by deep phenotyping including neuropsychological and ancillary investigations. Candidate variants are analyzed according to published phenotype-genotype correlations. In case of identification of a potential new pathogenic variant research project are undergone for validation of the new candidate. Adapted from Pipis et al, 2019.

WOLE EXOME SEQUENCING (WES)

WES technology performs analysis of protein encoding region in 85% of disease-causing mutations of rare Mendelian disorders. Indeed, the targeted hybridization enriches exomes and the resulting genomic library is then, sequenced on a specialized platform (Figure 18). Actually, various databases are available for the filtering and annotation of sequences data and variants. Despite the good coverage of sequencing, around 96% of targeted and mappable exomes, there are some regions with off target capture effects as the GC-rich regions of exon 1¹⁸⁵.

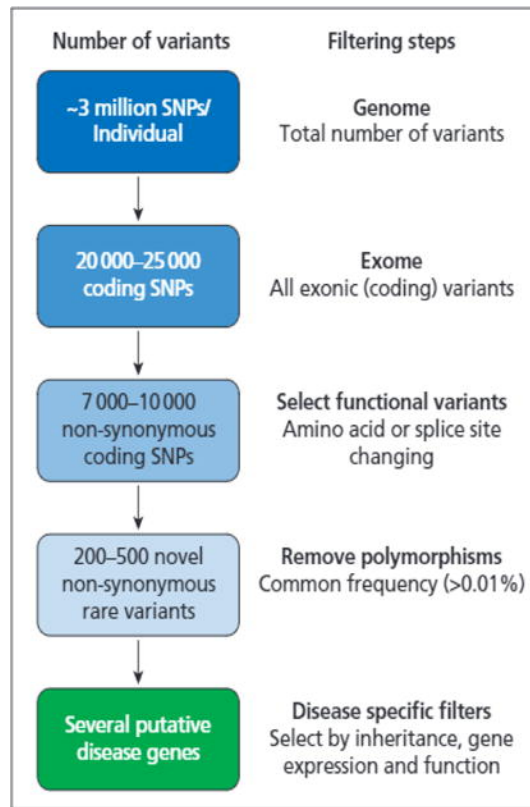


Figure 18 WES filtering and functional analysis technique.

The scheme illustrates an example of the WES analysis including sequences alignment, duplicates detection and data sequence and variant annotation. Adapted from Efthymiou et al 2016.

GENE PANELS

Custom-made panels have gain popularity in various diseases. The custom-made panels can include form 40 up to 160 genes are used in diagnostic purposes in specialized centers. The technique relies on solution hybridization that use pools of probes (biotinylated oligonucleotides) targeting a segment of the desired captured region in order to identify particular genome segments. This is a low-cost screening method for searching gene variants associated with specific disease¹⁸⁶.

ANNOTATION TOOLS AND ANALYSIS

VARAFT (VARIANT ANNOTATION AND FILTRATION TOOL)

VarAFT software allows annotation, filtering and breadth/depth coverage analysis in disease-causing mutations. This tool provides a full graphical interface and includes unique features to improve mutation annotation and prioritization. As mentioned, this software allows to combine small (single nucleotide variations, small insertions/deletions and large rearrangement (copy number variations) in order to analyze an individual genome. In order to allow variant detection from various data bases, under various formats including VCF/qVCF or tabulated files, it combines information from the dbNSFP, ANNOVAR including OMIM, HPO, Gene Ontology, pathways (Reactome, KEGG, PID) and predictions from UMD-Predictor, HSF (human Splicing Finder)¹⁸⁷.

GENOME-WIDE ASSOCIATION STUDIES (GWAS)

Linkage studies were classically used in order to confirm the association of a particular disease with specified genes. Recently, with the arrival of NGS technologies and the development of genome-based databases, allowed the development of GWAS.

GWAS is currently used in various diseases and also in neuromuscular disorders. In this approach the populations are separated into control and the disease sample. After performing sequencing in both populations, the quality control and annotation process the resulting SNP frequency variation will be analyzed according to the association to the trait of interest (Figure 19). In reality, the correlation of SNP with a pathological trait is not always possible. Various statistical measures are used in order to better identify the SNP and large number cohorts are necessary. In rare diseases, due to small number cohorts GWAS study are less obvious.

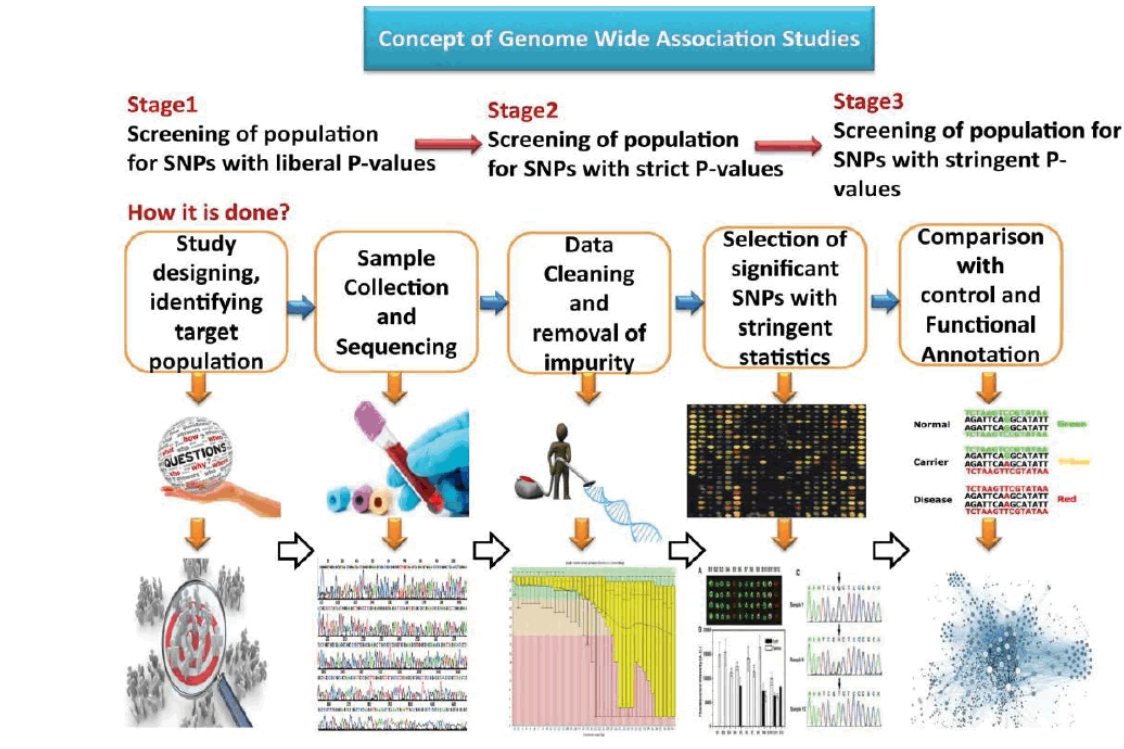


Figure 19 GWAS study method scheme illustration.

There 3 general stage of a GWAS analysis. The first stage includes the study design with population screening and sample collection form population of interest and control, followed by sequencing. In stage 2 the sequenced data is screened with based on stringent statistics and the last stage include the comparison and functional annotation of SNPs of interest. Abbreviations: GWAS=Genome Wide Association Studies, SNP=Single Nucleotide Polymorphism. Adapted from Banerjee et al, 2018.

In a recent study comparing WGS to WEG and mitochondrial sequencing and also gene panels the authors highlights the advantages of the WGS technique covering non-coding, coding and intergenic areas of nuclear and mitochondrial genomes. This work illustrates the utility of WGS not only in research purposes but also in diagnostic routine for rare disease conditions¹⁸².

Altogether, this summary of NGS and annotation tools are a small overview of the important advances in high-throughput DNA sequencing. These technical advances are largely used in the screening of neurological disorders and lately in DMD and BMD. Genotype-phenotype characterization implement these techniques in order to detect new pathological variants involved in disease progression and severity. A number of gene modifiers as the protective haplotype IAAM of *LTBP4* gene and TCTEX1D1 were recently discovered using the NGS technologies and demonstrating the feasibility of these techniques in DMD.

OBJECTIVES

The central dogma of therapy development in DMD independently of the applied strategy is to transform DMD patients in less severe BMD patients. Exon skipping in *DMD* gene in order to correct the reading frame and restore the dystrophin production has proven to be an efficient therapeutic solution for DMD patients. Indeed, increasing number of exon skipping therapies are currently FDA/EMA approved in DMD and currently there are two microdystrophin clinical trials ongoing. Despite the available FDA/EMA approved exon skipping drugs, the therapeutic choice is suitable only to a limited number of DMD patients. Moreover, encouraging preliminary results of microdystrophin clinical trials cannot answer to the questions of long-term efficacy because of a lack of a long-term data. Also, the caveats as the AAV personal immunity in young patients, large scale of AAV production and the dose dependent secondary adverse effects remain unsolved for the moment^{188,189}. Hopefully, the ongoing preclinical work on AAV modifications and also improvement in biotherapy protocols of vector large scale production and purification will help to solve current limitations.

Also, the development of alternative therapeutic strategies should be considered. Indeed, significant efforts with encouraging results have been achieved over the last decade in the development of multiple exon skipping (MES) therapies. As discussed above, several different approaches have already demonstrated proof-of-concept in MES by exon skipping or CRISPR/Cas9 systems. Based on literature data the MES of exons 45-55 could be eligible in 63% DMD patient with deletion mutations¹⁹⁰.

In addition to the increasing number of reported DMD /BMD patients that escape to the Monaco reading frame rule, phenotype variability within the same mutation can be reported. Indeed, the central dogma of transforming DMD in BMD assumes that BMD patients have a mild phenotype. Nonetheless, careful literature overview highlights major phenotype variability in BMD patients¹⁹¹⁻¹⁹⁵. More accurately, the BMD patients with exon 45 to 55 deletion have been classified as mild phenotype based on studies with small cohorts and published reports frequently compared DMD or BMD patients with different exon mutations^{69,196,197}. Furthermore, our group reported a phenotype variability in BMDdel45-55 patients with different degree of histopathological features.

In line with these data my PhD project proposes to characterize the BMDdel45-55 patients phenotype and genotype in order to predict therapeutic efficacy and identify new phenotype variability candidates. The following work will cover the results and the discussion based on the reported results in two separate parts. The first part will focus on phenotype and genotype characterization of the French cohort of BMDdel45-55 patients. The second part will target the study of lncRNA sequences localized in introns 44 and 56 and their involvement in myogenesis and their potential impact on phenotype of BMDdel45-55 patients.

AIM 1/ CHARACTERIZATION OF BMDDEL45-55 POPULATION

Advances in precision medicine by multiple exon-skipping (MES) 45 to 55 reported successful dystrophin gene mutation correction and protein expression in vitro but also in vivo in big animals^{67,137,145}. In humans, MES of exons 45-55 could be eligible in 62% of DMD deletion patients¹⁹⁰. Efficacy of any therapy approach can be deduced from the clinical features of patients with similar mutations. Surprisingly, there is no information that we know of the involvement of gene modifiers as *LTBP4*, *SPP1* and *Annexin* in BMD patients¹⁹⁸⁻²⁰¹. Also, in order to confirm the generality of the benign phenotype in BMDdel45-55 patients we initiated a retrospective analysis of French UMD-DMD data base in order to collect clinical data. In addition, we hypothesized that the genetic characterization should be taken beyond the whole-exome sequencing and we choose to perform whole genome sequencing in 19 BMDdel45-55. We questioned the presence of pathological variants in published cardiac and gene modifiers. Overall, this study aimed at depicting the complexity of the pathogenic processes and the genetic inter-individual variability.

AIM 2/ IDENTIFICATION OF NEW ACTORS OF PHENOTYPE VARIABILITY AND DYSTROPHIN REGULATION

lncRNA gain place in the regulatory actors of developmental myogenesis and regeneration^{166,202}. We sought to identify the presence and the impact of these noncoding sequences in the phenotype variability of BMDdel45-55 patients. First, we hypothesized that

the deletion breakpoint could impact the lncRNA sequences localized in introns 44 and 56. Then, based on the literature data on the downregulating role of these lncRNA on dystrophin promoter in DMD female carrier we hypothesized that the mechanism of action is in cis- and might be also involved in myogenesis, thus we tested the possible involvement of these lncRNA in proliferation and differentiation in human myoblasts. We hypothesized a different expression pattern in human myoblasts from healthy control compared to BMDdel45-55 and DMDdel45-52 patients. Overall, we sought to decipher the role of lncRNA in myogenesis and hoped to correlate the clinical phenotype of BMDdel45-55 to the presence and expression patterns of the lncRNA.

CHAPTER II: RESULTS

ARTICLE 1

Context:

As mentioned in thesis objectives and corresponding to Aim 1, we report in the first article entitled “Toward multi-exon skipping phenotype prediction through the genotype and phenotype characterization of the largest del45-55 Becker muscular dystrophy cohort” the results on clinical and genotype characterization of BMD Δ 45-55 patients.

Abstract:

The therapy development in DMD has been largely inspired by the clinical milder phenotype of BMD patients. Phenotype variability within the same mutation has been a major concern in MES development. In recent studies from our group ^{71,203} BMD Δ 45-55 patients were reported with various degree of severity. In order to study the genotype – phenotype correlation within this specific mutation cohort, we performed a retrospective analysis of French UMD-DMD data base and established the detailed phenotype characterization in 50 BMD Δ 45-55 patients.

Here we present the largest cohort of BMD Δ 45-55 and propose a clinical severity scale (CSS) for the retrospective description of the natural history evolution of the skeletal muscle presentation in this specific deletion. With the aim of investigating the phenotype variability in BMD patients within the same genetic mutation, we performed whole genome sequencing in 19 BMD Δ 45-55 in order to identify pathogenic variants in cardiac genes and in gene modifiers described in DMD.

This work is under preparation for publication submission.

Toward multi-exon skipping phenotype prediction through the genotype and phenotype characterization of the largest del45-55 Becker muscular dystrophy cohort

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ABSTRACT

Background: Duchenne and Becker muscular dystrophy (DMD and BMD) are X-linked myopathies characterized by a progressive muscular dystrophy with or without dilated cardiomyopathy (DCM). As described in literature, 63% of DMD patients are eligible to a multi-exon skipping therapy (MES), thus becoming BMD patients with exons 45 to 55 deletion (BMD Δ 45-55). The phenotype variability within the same genetic deletion is a major concern in the design of MES.

Objective: We conducted genotype and phenotype characterization of the largest BMD Δ 45-55 population.

Results: Here we present a population of 50 “skip-equivalent” BMD Δ 45-55 patients and study the phenotype variability by performing a detailed clinical characterization in 43 patients and assessing a whole genome sequencing analysis (WGS) in 19 patients. The phenotype characterization underlined that 100% patients have BMD phenotype and that, in patients with available cardiac investigation 30% had dilated cardiomyopathy (DCM). Using an in-house skeletal muscle clinical severity scale (n=43 patients) we revealed in 54% a mild clinical presentation. The WGS data analysis in 19 patients didn't identify the association of pathogenic variants in analyzed cardiac genes.

Conclusions: This study characterized the largest reported cohort of BMD Δ 45-55 patients and showed that, despite the exclusive BMD phenotype, different severity degree of skeletal muscle and cardiac presentation could be identified. This phenotype characterization confirming 100% BMD phenotype and genotype profiling in this specific mutation, supports that MES Δ 45-55 therapy could provide clinical benefit.

Key words: Becker muscular dystrophy, BMD, multi exon skipping, MES, phenotype variability, cardiomyopathy, lncRNA, genomic, gene modifiers

INTRODUCTION

Duchenne and Becker muscular dystrophies (DMD and BMD) are allelic disorders caused by pathogenic variants in *DMD* gene. The DMD incidence is about 1 in 3 000 - 5 000 new born boys and the clinical presentation is characterized by a rapid motor decline in early childhood and the loss of ambulation before 13 years of age with a shortened life span due to cardiac and respiratory complications¹⁻³. The onset of the disease in BMD patients was most frequently reported as starting at adult age, with an incidence of 1 in 18 000 - 30 000

live male births^{1,4,5}. The clinical symptoms of BMD were first described in 1955 as a benign variant of DMD⁶. A defining feature of BMD is a large spectrum of symptoms, including onset, severity and correlation between symptoms, and a heterogeneous rate of disease interindividual progression⁴. The BMD patients are ranging from asymptomatic to suffering from progressive loss of muscle mass and function, with some patients presenting isolated dilatative cardiomyopathy and/or DMD like phenotypes^{1,7}.

The main difference between DMD and BMD genotypes is whether the mutation in *DMD* gene is resulting in a mRNA that maintains the reading frame and allows the translation of a functional amino- and carboxy-frame⁸. In DMD patients the out-of-frame mutations result in the absence of this protein involved in the formation and maintenance of healthy muscle. The BMD-causing mutation leads to an abnormal, shorter dystrophin protein that is partially functional resulting in the more benign phenotype. Due to this particular feature the therapy dogma is to transform DMD patients into BMD patients. Different strategies have been developed and tested over the decades and one of the most promising approach remains exon skipping by inducing the skipping of central rod domain exons, restoring the reading frame^{9,10-12}. Currently 13% of DMD patients are eligible to FDA and EMA approved exon skipping oligo-antisense (OAN) therapies. In order to cover a larger number of DMD patients the multiple exon skipping (MES) appeared as an attractive alternative. The MES feasibility by OAN was demonstrated in preclinical models and recently CRISPR-Cas9 MES revealed encouraging results in systemic injection via viral vehicle¹³⁻¹⁶.

The choice of the skipping exons is ruled both by the efficacy of the skipping and the predictable clinical outcome. Among various mutations in *DMD* gene deletions

cover 60% of both DMD and BMD. Interestingly, 63% of DMD deletion patients are eligible to MES of exons 45 to 55 thus becoming BMD Δ 45-55 patients¹⁷. There is limited data about the natural history of BMD patients with the specific exon 45 to 55 deletion (BMD Δ 45-55). The BMD Δ 45-55 patients were reported as a benign skeletal muscle phenotype. Nevertheless, some documented patients with benign skeletal muscle clinical phenotype manifest severe dilatative cardiomyopathy (DCM) requiring cardiac transplant. The phenotype variability within the same genetic deletion is a major concern in the design of MES 45-55. There is scarce evidence of the etiology of mutation specific variability in BMD patients because many published cohorts combine various type of in-frame mutations^{4,18,19}. The published literature is based on small number of patients with this specific mutation or well documented, but limited number of patient case reports²⁰⁻²³. In addition, previous reports have described a high rate of "exceptions" to the reading frame rule with out-of-frame BMD. The positive predictive value of an in-frame mutation in UMD-DMD database was unexpectedly low, as it appears to be confirmed in 56-91%^{3,19,24,25}.

In a recent report authors analyzed the differences in the structure of mutant dystrophin and hypothesized that it may be responsible for the clinical heterogeneity

observed in BMD patients. Molecular homology modelling of the proteins corresponding to in-frame deletions in the rod domain close to the exons 45-55 ($\Delta 45-51$) have displayed a similar structure (hybrid repeat) comparing to the wild-type dystrophin²⁶. This data suggest that dystrophin protein structure may not be the only factor involved in phenotype variability in BMD patients. Moreover, gene modifiers as *LTBP4*, *SPP1*, *THBS1*, *Anxa6*, *ACTN3*, *TGFBR2*, *TCTEXD1* have been described in DMD as important actors in phenotype variability as protective haplotype (IAAM) of *LTBP4* in the loss of ambulation (LoA) or steroid treatments response²⁷⁻³⁴. Surprisingly, despite the great phenotype variability in BMD patients there is no information about the involvement of these candidates in BMD patients within the same genetic mutation. In order to confirm the generality of the benign phenotype in BMD $\Delta 45-55$ patients we performed retrospective analysis of

French UMD-DMD data base and established the detailed phenotype characterization in 50 BMD patients. Here we present the largest cohort of BMD $\Delta 45-55$ and propose a clinical severity scale (CSS) for the retrospective description of the natural history evolution of the skeletal muscle presentation in this specific deletion. With the aim of investigating the phenotype variability in BMD patients within the same genetic mutation, we performed whole genome sequencing in 19 BMD $\Delta 45-55$ in order to identify pathogenic variants in cardiac genes and in gene modifiers described in DMD. This work highlighted the utility of a new CSS score as an intuitive and versatile tool in the phenotype characterization and disease progression of BMD $\Delta 45-55$ patients. Phenotypic and genotypic data confirmed the benign phenotype of BMD $\Delta 45-55$ patients that could be beneficial for MES 44-45 for DMD.

METHODS AND POPULATION

Population characterization

The cohort included male Becker patients with an exclusive deletion mutation of exons 45 to 55. The population is taken from the French UMD-DMD database. The phenotype characterization was performed retrospectively from the medical records. Written consent was obtained for the diagnostic purposes. All participants were assigned de-identified numbers. This study was reviewed and approved by the French Institutional Review Board.

Retrospectively collected phenotype data included skeletal muscle symptoms and deficit if present, age at first skeletal muscle signs, age at last clinical examination, age at diagnosis, report/last follow-up if available, loss of ambulation (LoA) and age at LoA, cardiomyopathy (history, age at diagnosis, first and last available left ventricular ejection fraction, nocturnal ventilation (history, age at initiation), biopsy (history, result of Western blot); Creatinine kinase blood level at initial and last available evaluation, cognitive impairment in adult patients (>18y.o.) was considered as normal if no specific comments in medical file.

Cardiac evaluation

Cardiomyopathy was defined by a left ventricle ejection fraction (LVEF) \leq 45%.

Electrocardiograms were not used to define cardiomyopathy. The time in which abnormal cardiac values were first reported established the “onset” of cardiomyopathy. The age corresponding to the “onset” of cardiomyopathy was the youngest reported age when cardiac parameters met the definition of cardiomyopathy. When available, treatment protocol and also cardiac transplant information was recorded from medical files. The age of non-cardiomyopathic patients corresponds to the oldest reported age with available cardiac findings. Patients under 20 years old with normal cardiac parameters were excluded from the analysis.

Clinical Severity Scale (CSS)

In order to assess this evaluation, it is required to establish the age of the last follow-up date and the age of first clinical signs. We decided to make three categories of skeletal muscle complains: in the first category were included all the symptoms except muscular deficit as cramps, myalgia, fatigue, effort intolerance, episodes of myoglobinuria and elevated isolated CK. In the second category were included patients with skeletal muscle deficit and the third one the patients who lost ambulation (LoA). The score rules are as follows: if there are no complains (type 4) the patient is classified as asymptomatic Becker muscular deficit (ABMD), the presence of only symptoms is evaluated by the

or accompanied with symptoms he will be assigned into the intermediate type 2 (IBMD) and the severe type 1 (SBMD) the patients who lost partial or total ambulation. All patients with available clinical data in order to perform the CSS scoring were assigned into a specific type, in order to address the disease progression evaluation, we excluded patients of age < 20 years as we considered that there was not enough information about the disease progression.

CSS inter-operator and intra-operator variability

In order to address the issue of variability during the assessment of the CSS we performed blinded scoring of CSS by the same operator and by two different operators. The intra-operator variability is very low with 100 % of reproducibility and inter-operator reproducibility is 100%.

Bioinformatic analysis

The whole genome sequencing was performed in Centre National du Génotypage, on DNA extracted from lymphocytes isolated from blood samples of 30 patients. Libraries were prepared using the TruSeq DNA PCR-Free Library Preparation Kit (Illumina, San Diego, CA, USA), in order to reduce library bias and to enable a more homogeneous coverage of the genome in all regions, including regions which can be more difficult to sequence

HiSeq2000 as paired-end 100 bp reads. In order to obtain a 30X coverage, 3 lanes of a HiSeq2000 flow cell were required for each sample.

Bioinformatic analyses will be carried out by the “Genetics and Bioinformatics” team (Christophe Bérout) and dedicated bioinformaticians at INSERM UMR_S910 (Nicolas Lévy).

The bioinformatic analysis of each patient sequencing reads were performed by VarAFT Annotation and Filter for the variant analyses. VarAFT provided experiments quality, annotation and filtration of VCF files. Moreover, only variants in 65 genes implied in cardiomyopathies from the CARDIOGEN National Health Sector were selected through bioinformatics pipeline.

Variants showing concordant genotype were selected. The criteria selecting a SNP as a genetic candidate for the cardiac gene analysis modifier were: exonic and splicing non-synonymous SNPs; frequency rate <1%. The selected criteria were: abnormality of cardiovascular system morphology, abnormality of the cardiovascular system and dilated cardiomyopathy. The selected SNPs were filtered out with: probable, low or unknown pathogenicity and intronic, non-coding localization. Then, variants were classified

with regards to American College of Medical Genetics and Genomics (ACMG) criteria (Richards et al, 2015), with help of several prediction tools. Also, frequency of rare variants in our local in-house database was used for interpretation.

Statistical analysis

The statistical analysis was performed in Python and GraphPad Prism 8 software. Comparative analysis was calculated by Student's t test (two-tailed and unpaired, significance $p < 0.05$) for two groups comparison and in more than two groups, ordinary two-way variance analysis (ANOVA). Bioinformatic analysis of WGS data comparison between Intermediate/IBMD and Severe /SBMD

group was performed by Fisher Test (statistical significance $p < 0.05$).

Missing data management

Taking into consideration the retrospective nature of the study, we were facing a considerable amount of missing clinical data. In order to compensate this missing data, in addition to the clinical description extracted from the UMD-DMD data base, we have accessed the clinical files in Cochin hospital archive and contacted the medical doctors from the French Rare Health Care for Neuromuscular Diseases Network (FILNEMUS) in charge of the follow up of the included patients to collect additional information.

RESULTS

Characterization of the BMD Δ 45-55 cohort

We collected retrospectively the phenotype characterization from medical records for 50 patients. Venn diagram from Figure 1A illustrates the assessments reported here in BMD Δ 45-55 population. From a total number of 50 patients, Clinical Severity Scale (CSS) (Figure 1B) assessed in 43 patients allowed to characterized the skeletal muscle impairment. All patients had exclusive Becker phenotype and the median age at last examination (n = 50) was 47 years (range from 11-88; SD 23.9). Actual age distribution is shown in Figure 2. In this cohort the age at first clinical signs was reported to start in early childhood (<16years old) in 73%. Data from 9 family cases were obtained, it has to be noted that one patient had positive family history but we could not collect the clinical data except for the proband.

From the reported complains, the most disabling were the walking/running difficulties (44 %) and in 16% cases the dilatative cardiomyopathy. Pulmonary functional explorations and assisted ventilation data were available in limited number (n=23) and only two patients required nocturnal ventilation for obstructive apnea. Cognitive involvement was reported only in one patient and consequently, in adult patients, we considered that the lack of cognitive involvement specification in the medical files presumed its absence. Only 4% of loss of ambulation (LoA) despite the present skeletal symptoms (med=66yo). Fatigue and effort intolerance were present in 48%, myalgia and cramps in 38%, rhabdomyolysis in 45 % and one patient undergone a cardiac transplant despite a mild phenotype (Table 1).

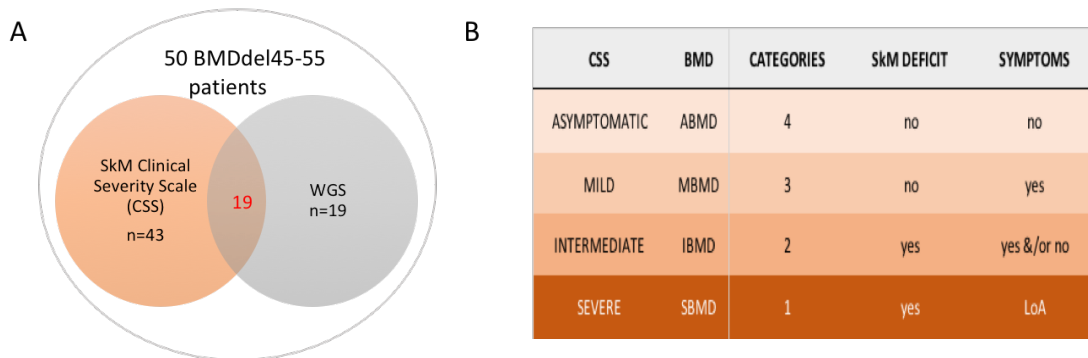


Figure 1 (A) BMD Δ 45-55 cohort description (n=50) Venn diagram of retrospective clinical characterization, skeletal muscle phenotype by the Clinical Severity Scale and Whole Genome Sequencing (WGS). (B) Clinical Severity Scale (CSS) of skeletal muscle evaluation in BMD Δ 45-55. Abbreviations: SkM= skeletal muscle, LoA= loss of ambulation, CSS=clinical severity scale; BMD= Becker muscular dystrophy, ABMD= asymptomatic BMD, MBMD= mild BMD, IBMD= intermediate BMD, SBMD= severe BMD

Diagnosis (Dx)		Skeletal muscle (SKM) evaluation										Cardiac evaluation					Pulmonary evaluation			Cognitif impairment										
patient number	Clinical Severity Scale (CSS)	age 1st SKM signs UN/Ddb (years)	age 1st SKM signs	age /last SKM examinatio n	cramps / SKM pain	fatigue/ effort intolerance	rhabdomyo lisis	cramps/ effort intolerance / fatigue (CEIF)	age CEIF (years)	walking/ running /ms deficit (WRMD)	age WRMD (years)	LoA	age LoA (years)	Ambulation (yes/no)/ Age (years)	Boston Score	Walton Score	CK value (Xfold of change)	Cardiac US: LVEF (%)	age last cardiac US	ECG anomalies (yes/no)	Cardiac transplant (yes/no)	Cardiac treatment (yes/no)	Pulmonary vital capacity (relative value %)	age pulmonary vital capacity detection (years)	Assisted ventilation (yes/no)	yes/no				
1	INTERMEDIATE	42	28	42	no	no	no	yes	2	yes	28	no	UK	yes	3	4	3	62	49	no	no	no	no	115	41	no	no			
2	INTERMEDIATE	40	40	49	yes	yes	no	yes	2	yes	40	no	UK	yes	2	3	2	66	54	no	no	no	no	113	UK	no	no			
3	MILD	8	8	19	yes	no	no	yes	19	no	NA	no	UK	yes	UK	2	13	UK	UK	UK	UK	UK	UK	120	5	UK	no			
4	MILD	UK	UK	no	yes	no	no	yes	UK	no	UK	no	UK	yes	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	no		
5	ASYMPTOMATIC	78	78	78	no	no	no	no	NA	no	NA	no	UK	yes	UK	UK	UK	65	78	UK	no	no	no	UK	UK	UK	no			
6	UK	UK	UK	UK	no	no	no	UK	UK	UK	UK	no	UK	yes	UK	2	4	UK	UK	UK	UK	no	no	UK	UK	UK	no			
7	MILD	56	53	56	yes	yes	yes	yes	53	no	UK	no	UK	yes	UK	2	4	UK	UK	UK	UK	no	no	UK	UK	UK	no			
8	MILD	1	1	9	no	no	no	yes	1	no	UK	no	UK	yes	UK	UK	25	65	9	no	no	no	no	UK	UK	no	no			
9	MILD	33,36	30	30	yes	no	no	yes	30	no	UK	no	UK	yes	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	no	no			
10	INTERMEDIATE	45	5	46	no	no	no	yes	5	yes	43	no	UK	yes	2	3	UK	45	46	UK	no	yes	91	UK	no	no	no			
11	SEVERE	61	2	53	yes	no	no	yes	2	yes	53	yes	60	UK	UK	7	1.5	41	53	yes	no	yes	105	52	no	no	no			
12	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	UK	no	no		
13	INTERMEDIATE	UK	48	59	no	no	no	no	UK	yes	48	no	UK	yes	UK	UK	UK	65	59	no	no	no	no	100	UK	yes	no			
14	INTERMEDIATE	5	5	41	no	yes	yes	yes	5	yes	34	no	UK	yes	2	3	16	50	47	no	no	yes	108	UK	no	no	no			
15	MILD	32	32	38	no	yes	no	yes	32	no	UK	no	UK	yes	UK	UK	UK	56	32	UK	no	no	UK	100	UK	no	no			
16	INTERMEDIATE	12	12	40	yes	no	yes	yes	7	yes	20	no	UK	yes	UK	UK	UK	UK	UK	UK	UK	no	no	100	UK	no	no			
17	INTERMEDIATE	UK	38	UK	no	no	no	UK	UK	yes	38	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	no	no	no		
18	INTERMEDIATE	UK	UK	UK	UK	UK	UK	yes	UK	yes	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	UK	no	no		
19	SEVERE	73	4	72	no	no	no	yes	4	yes	37	yes	72	no	UK	3	2	45	72	UK	no	yes	90	60	no	no	no			
20	UK	13	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	UK	no	no		
21	INTERMEDIATE	55	4	55	no	yes	no	yes	4	yes	40	no	NA	yes	UK	UK	2	44	52	UK	no	no	UK	UK	UK	no	no	no		
22	MILD	22	12	22	yes	yes	no	yes	12	no	UK	UK	UK	yes	1	1	10	UK	UK	UK	UK	no	no	UK	UK	no	no	no		
23	MILD	15	15	32	no	no	yes	yes	15	no	UK	no	NA	yes	UK	UK	UK	55	32	UK	no	no	no	UK	UK	no	no	no		
24	MILD	6,08	5	16	yes	yes	yes	yes	5	no	UK	UK	UK	yes	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	UK	no	no		
25	INTERMEDIATE	1	40	40	no	no	no	yes	1	yes	40	UK	UK	yes	UK	UK	6	UK	UK	UK	UK	no	no	UK	UK	UK	no	no		
26	INTERMEDIATE	40	40	40	no	no	no	no	UK	yes	40	no	UK	yes	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	UK	no	no		
27	INTERMEDIATE	53,6	42	42	no	no	no	UK	UK	yes	42	UK	UK	yes	2	3	1	40	62	yes	no	yes	UK	UK	UK	no	no	no		
28	UK	UK	50	50	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	UK	no	no	no	
29	INTERMEDIATE	11	11	62	yes	no	no	yes	11	yes	11	no	UK	yes	UK	UK	2	65	55	no	no	yes	UK	UK	UK	no	no	no		
30	INTERMEDIATE	10	50	50	no	no	no	no	NA	yes	50	UK	UK	yes	2	3	3	UK	UK	UK	no	no	UK	153	55	UK	no	no		
31	MILD	9,87	5	17	no	no	yes	yes	5	UK	UK	UK	UK	yes	1	1	UK	65	UK	no	no	yes	UK	UK	UK	no	no	no		
32	MILD	UK	UK	40	no	no	no	yes	40	no	UK	no	UK	yes	UK	UK	UK	35	40	UK	yes	UK	UK	UK	UK	UK	no	no		
33	MILD	12	12	20	yes	no	no	yes	12	no	UK	no	UK	yes	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	UK	no	no	no	
34	MILD	24	23	24	yes	yes	yes	yes	23	no	NA	no	UK	yes	1	1	6	65	24	no	no	no	no	UK	UK	no	no	no		
35	INTERMEDIATE	75,83	6	76	yes	yes	yes	yes	5	yes	75	UK	UK	yes	UK	UK	10	UK	UK	no	no	no	no	73	69	no	no	no		
36	INTERMEDIATE	4,22	4	4,22	yes	yes	yes	yes	4	yes	4	no	UK	yes	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	UK	no	no	no	
37	INTERMEDIATE	4	4	6	yes	yes	yes	yes	4	yes	4	no	UK	yes	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	UK	no	no	no	
38	INTERMEDIATE	4	4	6	yes	yes	yes	yes	4	yes	4	no	UK	yes	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	UK	no	no	no	
39	MILD	3	UK	UK	no	yes	yes	yes	3	no	UK	no	UK	yes	UK	UK	10	66	19	UK	no	no	UK	UK	UK	UK	yes	yes		
40	MILD	12,76	7	12,76	no	yes	yes	yes	7	UK	UK	UK	UK	yes	UK	1	UK	60	UK	UK	UK	no	no	UK	UK	UK	no	no	no	
41	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	yes	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	UK	no	no	no	
42	MILD	14,59	12	33	yes	yes	yes	yes	12	no	UK	UK	UK	yes	1	40	65	30	UK	no	no	no	no	UK	UK	UK	no	no	no	
43	MILD	25	25	34	no	no	yes	yes	25	no	NA	no	UK	yes	UK	5	63	30	UK	no	no	yes	UK	UK	UK	no	no	no	no	
44	MILD	35	UK	35	yes	yes	yes	yes	25	no	NA	no	UK	yes	UK	UK	5	45	50	no	no	yes	UK	UK	UK	no	no	no	no	
45	MILD	11	12	14	yes	yes	no	yes	12	no	NA	UK	UK	yes	1	1	7	65	UK	UK	no	no	UK	UK	UK	no	no	no	no	
46	INTERMEDIATE	7,82	7	8	no	no	no	yes	7	UK	yes	7	UK	yes	1	1	26	UK	UK	UK	no	no	no	UK	UK	no	no	no	no	
47	INTERMEDIATE	40	6	65	no	no	yes	yes	20	yes	62	no	UK	yes	UK	UK	10	62	66	UK	no	no	UK	UK	UK	no	no	no	no	
48	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	UK	yes	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	UK	no	no	no	no
49	UK	10	10	10	UK	UK	UK	UK	UK	UK	UK	UK	UK	yes	UK	UK	UK	UK	UK	UK	UK	no	no	UK	UK	UK	no	no	no	no
50	MILD	6	6	10	no	no	yes	yes	6	no	UK	UK	UK	yes	UK	UK	30	UK	UK	UK	no	no	UK	UK	UK	UK	no	no	no	no

Table 1 Clinical features of BMD45-55 patients (n=50). Abbreviations: del=deletion, Dx=diagnostic, LoA= loss of ambulation, CK= creatine kinase, UI=international units, blanc cells= data not reported in medical files, color lines= family case, CEIF=cramps, effort intolerance, WRMD=walking/running deficit

New clinical severity scale (CSS) for BMD Δ 45-55 patient stratification

In order to harmonize the skeletal muscle clinical evaluation, a new scale of clinical severity (clinical severity scale (CSS)) was proposed. Indeed, this scale could be used in order to better stratify patients according to the progressive character of the Becker muscular dystrophy. BMD Δ 45-55 patient's clinical characterization was classified by CSS in four types, according to their skeletal muscle symptoms, deficit and loss of ambulation (LoA). The severe BMD Δ 45-

55 (SBMD) characterized by LoA was classified as type 1, the intermediate (IBMD, type 2) indicates the presence of skeletal muscle deficit or walking/running muscular deficit (MD), the mild (MBMD, type 3) denote the presence of symptoms as fatigue, effort intolerance, cramps and transient episodes of myoglobinuria without skeletal muscular deficit and the asymptomatic (ABMD) as type 4 with absent complains (Figure 1B, see methods for more details). In the following we report the CSS of the BMD Δ 45-55 patient cohort.

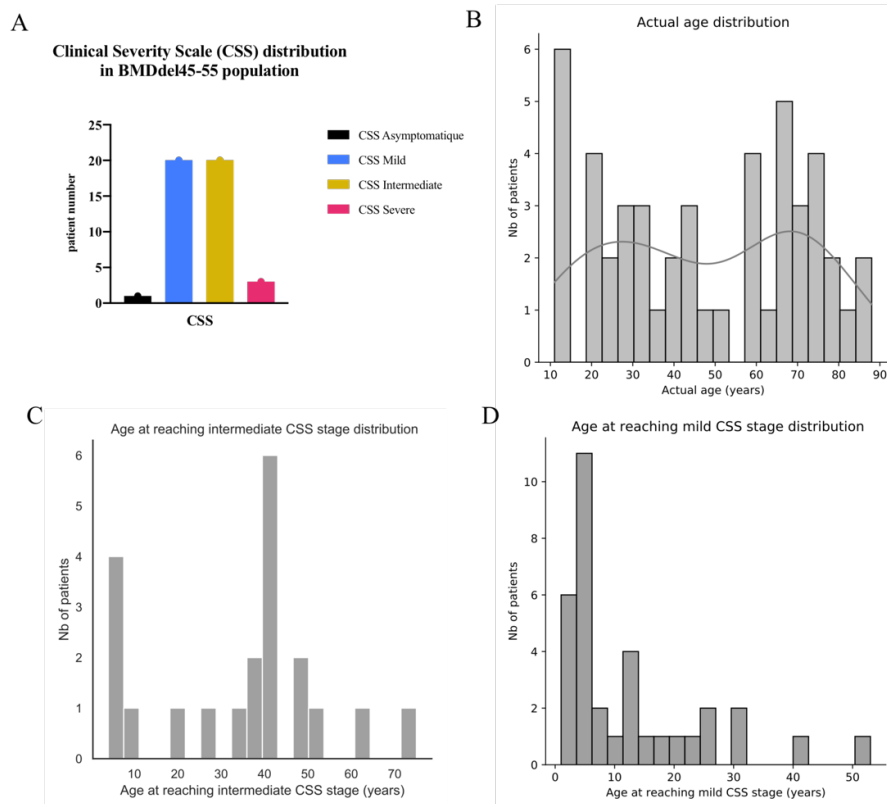


Figure 2 Clinical Severity Scale (CSS, n=43) and age distribution in BMD Δ 45-55 population (A) CSS distribution in 43 patients (B) Distribution of the current age (C) Age distribution at reaching Intermediate CSS (D) Age distribution at reaching Mild CSS

Surprisingly given the literature data, only one patient was classified as asymptomatic

and 2 patients were identified with severe clinical type. The asymptomatic patient N5

did not report any skeletal muscle complains until the age of 78 with occasional fatigue. Patient N11 and N19 reported first clinical signs in little infancy (4 y.o.) with delayed age of walking acquisition and effort intolerance symptoms. Patient N11 started to have skeletal muscle deficit at 50 years old (y.o.) and during his last examination at age 53 y.o. the Walton score was at 7, suggesting a rapid progression of dystrophy and clinical phenotype severity. He started to use the wheel chair (LoA) at age 60y.o. and the CK levels were reported 1,5x. The second Severe patient N19 reported his first skeletal muscle deficit signs at 40 y.o., during the last clinical examination at 72 the muscular force evaluation confirmed muscular weakness in lower limbs (evaluated according to MRC) with quadriceps hypotrophy. The LoA was reported at age 72. CK levels were at 2x. Compared to the Severe patient n11 the disease initiation was earlier and disease progression was slower suggesting different factors involved in dystrophy progression despite the same exonic deletion. The age of diagnosis was reported at 4 y.o. Unfortunately, for both patients with SBMD the dystrophin evaluation was not reported in medical files and probably not performed at diagnosis.

In 20 patients classified as IBMD, type 2, the average age at first signs was 5.7+-4.9, a first skeletal muscle deficit symptoms

were identified on average at 33.1+-20 and the mean age at the last examination was 40.6+-21. The Boston scale, when available, was scored at 2 in 71 % of patients, and the Walton scale 3 in 71% as well. CK values ranged from 2-16x.

In 20 patients with MBMD, type 3, the median age at first symptoms were reported at 17.7+-14 and the mean age at last examination was 26+-12.5. The main declared symptoms were fatigue in 30%, effort intolerance in 15%, myoglobinuria in 15% and myalgia in 30%. Reported CK levels ranged from 4x to 10x.

Disease progression by reaching mild, intermediate and severe type of CSS

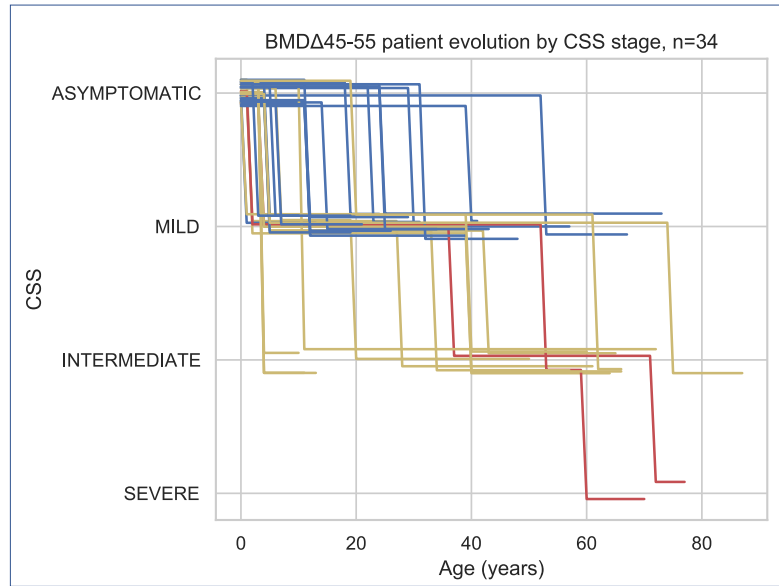
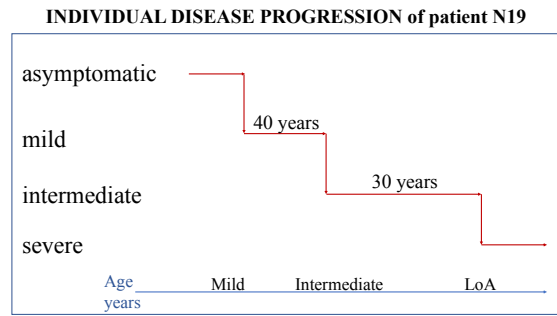
Disease progression of the of 34 patients was depicted according to their age reaching each CSS stage in a figure inspired by the survival Kaplan-Meier plot (Figure 3A). As an example, a SBMD patient individual disease progression is illustrated in figure 3A. His age was reported when reaching the CEIF symptoms, muscular deficit (WRMD) and ultimately the partial loss of ambulation (LoA). The retrospectively estimated time free of skeletal muscle deficit was 40 years and 30 years before the partial loss of ambulation respectively patient N11 and N19.

In the majority of mild patients (MBMD) the disease begins in late childhood. In the intermediate patients the initial stage of the disease features only symptoms and

progressively they develop skeletal muscle deficit. Interestingly, the age of reaching the mild stage (age at CEIF) in intermediate group of patients is very early, below the age of 7 y.o. in 11 cases. Also, a family case of free brothers had inaugural skeletal muscle deficit at age of five. Only two patients were categorized as severe and they had first clinical signs at age 5 and featured skeletal muscle deficit (age at WRMD) for an average of 35 years before losing the ambulation. This data confirms the slowly progressive character of muscular dystrophy and also indicates an association

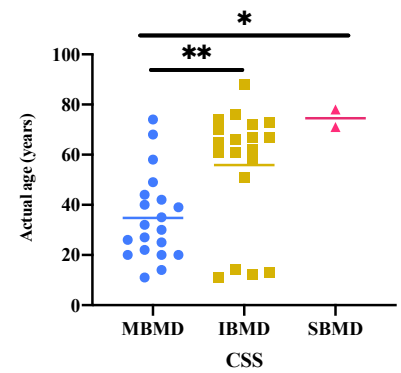
of the age to the CSS scale. Indeed, we report a significative difference between the actual age of patients in MBMD comparing to IBMD ($p=0.005$) and to SBMD ($p=0.02$) (Figure 3B). Interestingly, CK level was correlated to the CSS severity (Figure 3C). Moreover, there is a significant difference in the age of reaching the MBMD compared to actual CSS stage ($p=0.01$) (Figure3D). The limited number of patients with SBMD did not allowed to reach statistical significance in the analysis of association of age at reaching WRMD compared to the actual CSS scale.

A



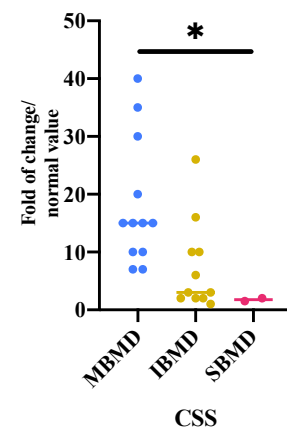
B

Actual BMD Δ 45-55 patients age vs CSS stage



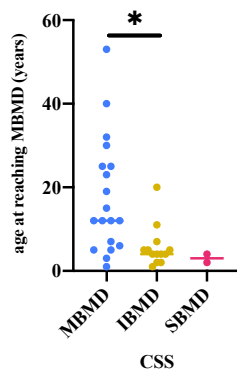
C

CK at Dx vs current CSS stage



D

Age at reaching MBMD vs current CSS stage



Age at reaching IBMD vs current CSS stage

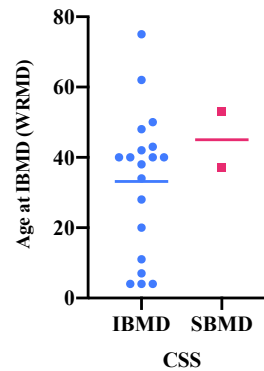


Figure 3 Disease progression according to CSS in BMD Δ 45-55 patients. (A) Kaplan-Meier inspired representation of individual disease progression for patient N19, with age at reaching Mild CSS (CEIF), at Intermediate CSS (WRMD). Visualization of disease progression in 34 individual BMD Δ 45-55 patient; blue lines illustrate the Mild CSS patients, yellow the Intermediate CSS and red the Severe CSS patients, (B) Actual age associated to the current CSS stage (C) CK at diagnostic are associated by current CSS (D) Age at reaching the Mild and Intermediate CSS according to current CSS. Abbreviations: CEIF=cramps, effort intolerance, WRMD=walking/running deficit, MBMD=mild, IBMD=intermediate, SBMD=severe Becker muscular dystrophy, LVEF= left ventricular ejection fraction, CK=creatinase, Dx=diagnostic.

Cardiac phenotype of the BMD Δ 45-55 patients

A cardinal feature of phenotype variability in BMD is the presence of a cardiomyopathy and this data was evaluated by echocardiography in 50% (n=27) of reported patients. Moreover, in 33% of patients the diagnostic of cardiomyopathy was established. The median age at the cardiac evaluation was 45+/-17, and the majority of patients were under cardioprotective therapy, with the therapy protocol data missing in 3 patients. The LVEF in SBMD patients was performed at 53 and 73 y.o. respectively. From 26 patients with missing cardiac phenotype the median age was 37+/-20 and, in most cases, patients refused the medical follow-up. Based on initial evaluation it is possible that they have a mild skeletal muscle phenotype but this does not exclude the possibility to develop cardiomyopathy. Particularly, in this cohort, one patient N32 underwent cardiac transplantation at age of 40 and had a mild skeletal muscle phenotype. His first clinical signs were reported at age of 36 and he had a rapid cardiac deterioration. We report three distinct populations, the young patients with Mild CSS (MBMD) with normal (>45%) values, the population of Intermediate CSS (IBMD) along with the asymptomatic patients (normal LVEF values) and the population of pathologic

LVEF values with Intermediate and Severe CSS (IBMD and SBMD) (Figure 4A). Significant difference was determined between the LVEF (%) in SBMD compared to MBMD (Figure 4B). There was no significant correlation between the LVEF (%) vs CSS types nor the age of first clinical signs.

The Dystrophin protein level suggests disease rate of progression

Despite the retrospective nature of this study, we managed to obtain the diagnostic Western Blot (WB) analysis (Cochin APHP, Paris) for 11 previously reported (Gentil et al.) and 3 additional patients. Despite the fact that the WB assay is not a quantitative assay, we took advantage that the diagnostic WB was performed by the same laboratory technician in all patients and for consistency issue we performed the relative quantification on ImageJ by the same operator.

As expected, the IBMD had a high amount of dystrophin protein, in opposite to SBMD patient who had a more heterogeneous amounts of dystrophin (Figure 4 D). A negative correlation between age at first skeletal muscle symptoms and the quantification of the dystrophin relative deficit is reported (p=0.02) (Figure 4 D). Interestingly, this result suggests that independently of a high level of dystrophin

protein, young patients already had walking and running difficulties at 5 years old. Additional analysis with different type of in-frame and out-of-frame mutations in *DMD* gene could be necessary to confirm these results.

Overall, our results suggest that despite young age and high dystrophin quantity there are independent factors triggering the disease progression and functional deficit.

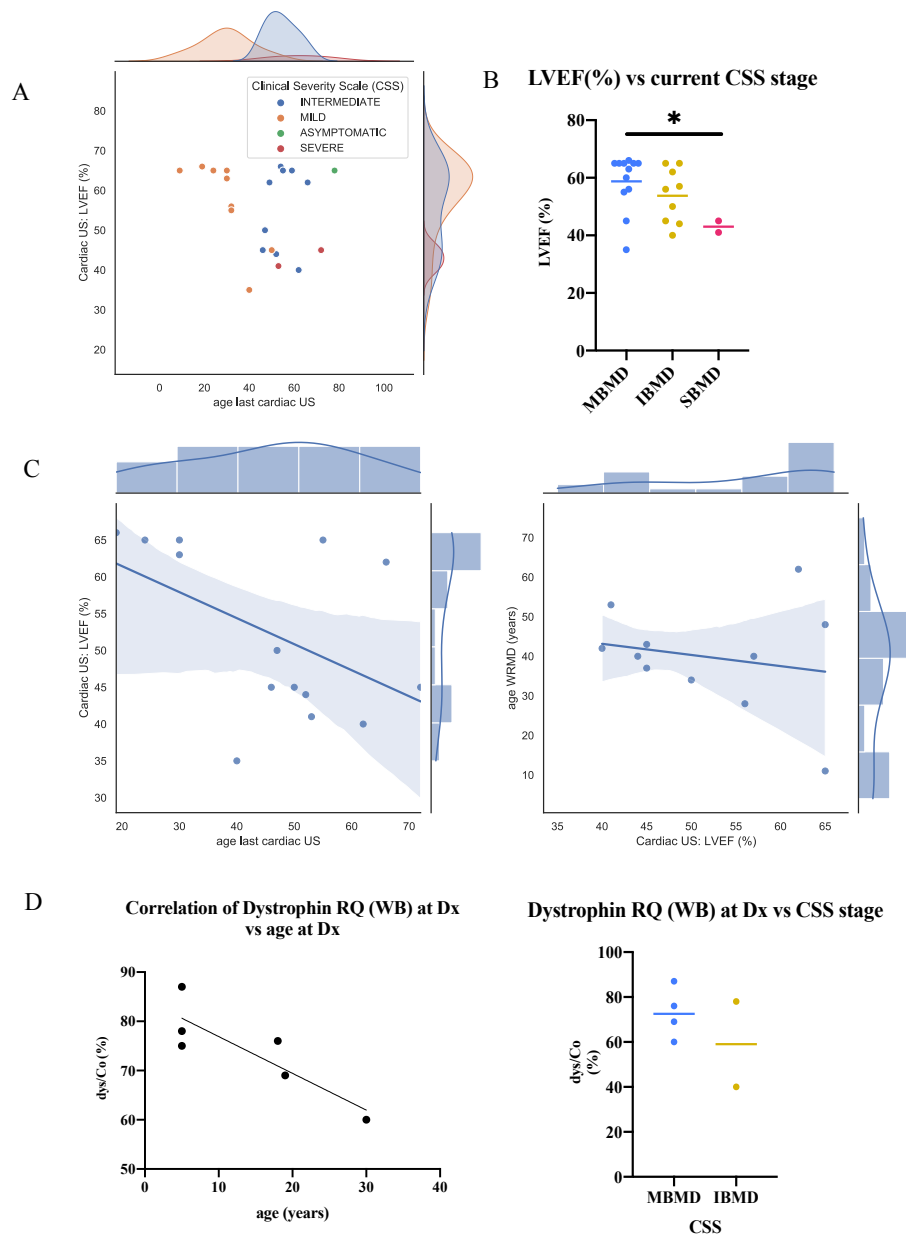


Figure 4 Cardiac exploration in BMD Δ 45-55 patients (n=23). (A) Scatter plot of cardiac functional evaluation by LVEF (%) by ultrasonography (US) against age at last cardiac evaluation colored according to CSS, (B) LVEF (%) correlation by current CSS (C) Scatter plot and correlation of cardiac LVEF against age at reaching MMD (D) Scatter plot and correlation of cardiac LVEF against age at reaching IBMD (D) Dystrophin analysis in BMD Δ 45-55 patients (n=14). (A) relative quantification (RQ) of dystrophin protein by Western Blot (Dystrophin/Coomassie) against CSS actual stage (B) correlation analysis between dystrophin RQ and the age at the muscular biopsies (p=0,02). Abbreviations: MBMD=mild, IBMD=intermediate, SBMD=severe Becker muscular dystrophy, LVEF= left ventricular ejection fraction, dys/Co (%) = dystrophin/ Coomassie, Pearson statistical test, R^2 p=0.02

Cardiac genotype characterization of the BMDA45-55 patients

We report a high number of cardiomyopathy (33%) in this mutation specific cohort. In order to address the cardiac phenotype variability issue, we performed WGS in 19 patients and analyzed the variants found in 65 cardiac genes from the CARDIOGEN list. First, we selected the candidate genetic modifiers corresponding to the established frequency threshold (<1%), exonic, close intronic location and pathogenicity prediction tool, UMD Predictor. In addition, we selected the following clinical criteria of: dilated cardiomyopathy, cardiac failure and hypertrophic cardiomyopathy (Ref). Then, variants were classified with regards to American College of Medical Genetics and Genomics (ACMG) criteria (Richards et al, 2015), with help of several prediction tools. Also, frequency of rare variants in our local in-house database was used for interpretation. Resulting SNP are listed in Table 2 and were classified based on ACMG criteria as follows: 1/benign, 2/probably benign, 3/unknown

significance,4/probably pathogenic, 5/pathogenic. Reported results did not reveal any pathogenic SNP.

None of the family cases share the same SNP variants. As expected, the majority of SNPs were found in the *TTN* gene but none of these variants had an ACMG score > 3/5. Then, followed *RBM20* and *FBMN1*, identified in two different patients each. Interestingly, variants within the same gene did not repeat even in the family cases (n=3). Interestingly, patient N19 with Severe CSS phenotype and cardiomyopathy only had one variant in BAG gene, but after detailed analysis this SNP was classified as benign. In contrary, for the patient 11, severe BMD with severe cardiomyopathy, the SNP in *FLNC* gene may represent a potential candidate for future studies, as this SNP is classified of unknown significance, mainly because of its low frequency. Based on reported WGS data, we confirm the utility of this sequencing in order to stratify the potential genes SNP association in cardiac pathology.

Patient no	CSS	LYEF(%)	Cardiomyopathy	Chr	Start	End	Ref	Alt	Func ref gene	Gene ref gene	Exon/Intronic	AA Change of gene	rs/prot	UVD Score	UVD Prediction	gnomAD genome	gnomAD exon	Yvarious	Yvarious criteria	Intovar	Intovar criteria	Clinvar	IntU(CSC) (local database)	Interpretation based on ACMG	ACMG Criteria
1	Intermediate	56	no	D=30/H0H01_AIVarants_VR.2	179425733	179425733	C	G	exonic	FNAC	nonsynonymous SNV	FNAC.NM_00319 exon154:c.163>155A/G409	163>155	Pathogenic	Pathogenic	1.63e-05	1.63e-05	US	PAC BP1	US	PAC BP1	11S	no	3	PM1 PM2 BP1
11	Severe	41	yes	D=30/H0H01_AIVarants_VR.2	128481523	128481523	C	T	exonic	FNAC	nonsynonymous SNV	FNAC.NM_00127487 exon133:c.133>134G/A	133>134	Pathogenic	Pathogenic	0.0017	0.0017	B	BP6	LP	BP6	1B4LBP7US	several panels	1	BS1 BS2
8	Mild	65	no	D=30/H0H01_AIVarants_VR.2	179425208	179425208	G	T	exonic	TTN	nonsynonymous SNV	TTN.NM_00319 exon146:c.146>147G/A	146>147	Pathogenic	Pathogenic	0.0002	0.0002	B	BP6	US	BP6	3LB**	no	2	PM1 PM2 BP1
19	Severe	45	yes	D=30/H0H01_AIVarants_VR.10	4871370	4871370	C	T	exonic	FN1	nonsynonymous SNV	FN1.NM_000133 exon2:c.76>77G/A	76>77	Pathogenic	Pathogenic	1.63e-05	1.63e-05	LP	BP6	US	BP6	no	3	PM1 PM2 BP1	
20	Severe	45	yes	D=30/H0H01_AIVarants_VR.10	121429634	121429634	G	A	exonic	BAG3	nonsynonymous SNV	BAG3.NM_004281 exon2:c.453>454G/A	453>454	Pathogenic	Pathogenic	2.62e-05	2.62e-05	LB	BP6	US	BP6	no	2	PM1 PM2 BP1	
21	Intermediate	44	yes	D=30/H0H01_AIVarants_VR.2	179441295	179441295	T	C	exonic	TTN	nonsynonymous SNV	TTN.NM_00319 exon153:c.153>154G/A	153>154	Pathogenic	Pathogenic	0.0076	0.0076	B	BP6	US	BP6	no	1	BS1 BS2 BP1	
21	Intermediate	44	yes	D=30/H0H01_AIVarants_VR.2	179418346	179418346	C	T	exonic	TTN	nonsynonymous SNV	TTN.NM_00319 exon162:c.162>163G/A	162>163	Probably pathogenic	Probably pathogenic	0.0013	0.0013	B	BP6	LP	BP6	1B4LBP6US	one panel	2	PM1 BS1 BP1
31	Mild	65	no	D=30/H0H01_AIVarants_VR.2	179428289	179428289	C	T	exonic	TTN	nonsynonymous SNV	TTN.NM_00319 exon2:c.205>206G/A	205>206	Pathogenic	Pathogenic	0.0068	0.0068	B	BP6	US	BP6	no	2	PP3 BS1 BP1	
31	Mild	65	no	D=30/H0H01_AIVarants_VR.2	37822318	37822318	C	T	exonic	TCAP	nonsynonymous SNV	TCAP.NM_003673 exon2:c.460>461G/A	460>461	Probably pathogenic	Probably pathogenic	0.0012	0.0012	B	BP6	US	BP6	no	3	PM2 PP3	
34	Mild	65	no	D=30/H0H01_AIVarants_VR.2	179481536	179481536	C	T	exonic	FN1	nonsynonymous SNV	FN1.NM_000133 exon29:c.378>379G/A	378>379	Pathogenic	Pathogenic	0.0052	0.0052	B	BP6	US	BP6	no	1	PM1 PM2 PP3	
42	Mild	63	no	D=30/H0H01_AIVarants_VR.2	179400130	179400130	G	A	exonic	NEKN	nonsynonymous SNV	NEKN.NM_00117239 exon109:c.109>110G/A	109>110	Pathogenic	Pathogenic	0.0002	0.0002	B	BP6	US	BP6	no	3	PM1 PP3 BS1	
35	Intermediate	-	yes	D=30/H0H01_AIVarants_VR.2	179403853	179403853	T	C	exonic	TTN	nonsynonymous SNV	TTN.NM_00319 exon181:c.181>182G/A	181>182	Pathogenic	Pathogenic	0.0002	0.0002	B	BP6	US	BP6	no	2	PM1 PP3 BS1	
35	Intermediate	-	yes	D=30/H0H01_AIVarants_VR.2	179434186	179434186	T	A	exonic	TTN	nonsynonymous SNV	TTN.NM_00319 exon154:c.154>155G/A	154>155	Pathogenic	Pathogenic	0.0002	0.0002	B	BP6	US	BP6	no	2	PM1 PP3 BS1	
34	Mild	65	no	D=30/H0H01_AIVarants_VR.2	179582913	179582913	C	T	exonic	TTN	nonsynonymous SNV	TTN.NM_133378 exon83:c.2105>2106G/A	2105>2106	Probably pathogenic	Probably pathogenic	0.0055	0.0055	B	BP6	US	BP6	no	1	BS1 BS2 BP1	
34	Mild	65	no	D=30/H0H01_AIVarants_VR.2	179486037	179486037	C	A	exonic	TTN	nonsynonymous SNV	TTN.NM_00319 exon74:c.132>133G/A	132>133	Pathogenic	Pathogenic	0.0052	0.0052	B	BP6	US	BP6	no	1	BS1 BS2 BP1	
42	Mild	63	no	D=30/H0H01_AIVarants_VR.2	179481536	179481536	C	T	exonic	TTN	nonsynonymous SNV	TTN.NM_00319 exon84:c.208>209G/A	208>209	Pathogenic	Pathogenic	5.72e-05	5.72e-05	LB	BP6	US	BP6	no	2	PM1 PP3 BS1	
36	Intermediate	-	-	D=30/H0H01_AIVarants_VR.2	179611809	179611809	-	-	exonic	TTN	nonframeshift insertion	TTN.NM_133378 exon46:c.153>155G/A	153>155	NA	NA	0.0027	0.0027	B	BP6	US	BP6	no	1	PM1 PP3 BS1	
36	Intermediate	-	-	D=30/H0H01_AIVarants_VR.2	179455352	179455352	C	T	exonic	TTN	nonsynonymous SNV	TTN.NM_00319 exon132:c.33>34G/A	33>34	Pathogenic	Pathogenic	0.0034	0.0034	B	BP6	US	BP6	no	1	PP3 BS1 BS2	
22	Mild	-	-	D=30/H0H01_AIVarants_VR.1	237754015	237754015	A	G	exonic	RYP2	nonsynonymous SNV	RYP2.NM_001053 exon31:c.316>317G/A	316>317	Probably pathogenic	Probably pathogenic	7.53e-05	7.53e-05	B	BP6	US	BP6	no	1	PM2 PP3 BS1	
24	Mild	-	-	D=30/H0H01_AIVarants_VR.10	75554210	75554210	C	T	exonic	VCL	nonsynonymous SNV	VCL.NM_003773 exon11:c.153>154G/A	153>154	Pathogenic	Pathogenic	5.30e-05	5.30e-05	B	BP6	US	BP6	no	1	BS1 BS2 BP1	
46	-	-	-	D=30/H0H01_AIVarants_VR.10	21097546	21097546	G	A	exonic	NEBL	nonsynonymous SNV	NEBL.NM_006393 exon26:c.26>27G/A	26>27	Probably pathogenic	Probably pathogenic	0.0024	0.0024	B	BP6	US	BP6	no	2	PM1 PP3 BS1	
45	Mild	65	no	D=30/H0H01_AIVarants_VR.2	39262348	39262348	C	G	exonic	SOS1	nonsynonymous SNV	SOS1.NM_006363 exon6:c.1074>1075G/A	1074>1075	NA	NA	0.0067	0.0067	B	BP6	US	BP6	no	1	BS1 BS2 BP1	
45	Mild	65	no	D=30/H0H01_AIVarants_VR.2	179449883	179449883	G	A	exonic	TTN	nonsynonymous SNV	TTN.NM_00319 exon138:c.37>38G/A	37>38	Pathogenic	Pathogenic	0.0022	0.0022	B	BP6	US	BP6	no	3	PM1 PM2 PP3	
45	Mild	65	no	D=30/H0H01_AIVarants_VR.1	3329229	3329229	G	C	exonic	PRDM16	nonsynonymous SNV	PRDM16.NM_022114 exon9:c.93>94G/A	93>94	Pathogenic	Pathogenic	0.0022	0.0022	B	BP6	US	BP6	no	1	BS1 BS2 BP1	

Table 2 Cardiac pathogenic variants in BMD45-55 cohort

Larger number of protective genetic modifiers in mild BMDA45-55 patients

Here we report the WGS analysis of genetic modifiers previously reported in DMD patients (*TCTEX1D1*, *ACTN3*, *THBS1*, *LTBP4*, *TGFBR2*, *SPPI*). From 19 patients screened by WGS only 18 featured SNPs in published genetic modifiers, shown in Table 3. The deleterious SNPs are illustrated in red and the protective ones in blue, and in order to take into consideration the progressive nature of the disease and the presence of the SNPs, the age distribution was highlighted by a grey scale. Overall, we highlighted a high variability in the presence of protective SNPs, from none to a maximum of eleven.

The deleterious *SPPI* SNPs was identified in the majority of patients with skeletal muscle deficit, also in one of the two severe patients. The *LTBP4* SNPs resulting in a deleterious haplotype T-VTTT was found in three patients, two relatives (p20 ♀ and p21 ♀), one with intermediate CSS phenotype and one for which clinical data were not sufficient in order to assign a skeletal muscle clinical severity score and one relatively young and currently mild patient. In contrary, the protective C-IAAM was found in a mild patient with an older age at first clinical signs.

The deleterious *TCTEX1D1* was found in only one intermediate patient altogether

with a protective SNP in *ACTN3*. This patient's first age of skeletal muscle symptoms was at 6 years old and the first muscular deficit was reported at a late stage around the age at 72 years. In addition, this patient's relative included in this cohort, featured only the protective SNP and reported the first skeletal muscle symptoms at age 23. Despite a relatively young actual age, 32 years, he is still free of skeletal muscle deficit suggesting a milder phenotype and a potential candidate for a long-term mild category.

The SNPs profile of genetic modifiers in the two severe patients are of a major interest. Indeed, one of the severe patients does not have any of deleterious SNPs and features two protective SNPs, however he lost ambulation at age of 60 years and features severe cardiomyopathy. The second severe patient has the deleterious *SPPI* SNP and the protective *TGFBR2* SNPS, indeed this patient has lost his ambulation at a very advanced (72 y.o.) age that could be explained by the protective SNPs presence that is supposed to alleviate his phenotype.

In the Figure 5 we depicted the SNP distribution according to the age of first skeletal muscle symptoms and CSS stage. Data showed that patients with early age at first skeletal muscle symptoms or deficit were found with the largest number of deleterious variants, and that patients with

mild severity CSS score tend to have the largest number of SNPs in various protective genetic modifiers.

highlight trends compatible with the described effects of the studied SNPs on phenotypes but with a high variability.

Altogether these results have allowed to depict a detailed map of various actors involved in phenotype variability that was never studied in BMD Δ 45-55 patients. We

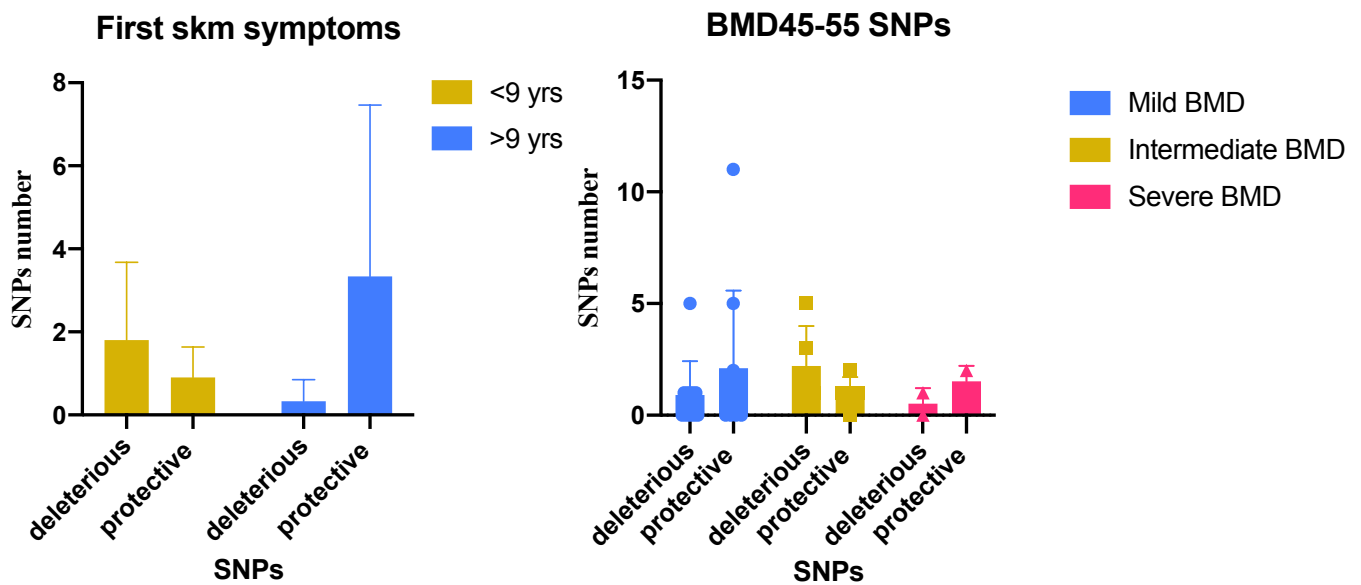


Figure 5 SNPs in genetic modifiers distribution. On the left side, SNPs represented according to the age of first skeletal muscle symptoms and at right, distribution according to the Clinical Severity Scale.

Gene	ID	REF	ALT	p24	p40	p8*	p45	p42	p34†	p22	p9*	p36	p31	p20	p46	p1	p21‡	p27	p35††	p11*	p19	Age
TCFEX1D1	rs1060575	A	I	5	7	9	12	12	23	12	30	4	5	uk	uk	2	4	uk	6	2	4	First skm symptoms
	rs3816989	G	A	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	uk	7	28	40	42	75	53	37	First skm deficit
	rs1815739	T	C	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	60	72	LoA
ACTN3	rs1004866	A	G	26	22	14	20	30	32	40	59	11	20	34	13	62	67	74	88	71	79	Actual age
	rs2725797	G	A																			
	rs2624259	C	T																			
THBS1	rs814518	G	A																			
	rs710160	T	C																			
	rs2303729	G	A																			
	rs1131620	A	G																			
	rs1051303	A	G																			
LTP4	rs10880	C	T																			
	rs4522809	A	G																			
	rs28357094	T	G																			
TGFBR2																						
SPP1																						
CSS																						
* †, †† ‡ family cases																						

Table 3 BMDdel45-55 patient genomic status of SNPs in several genetic modifiers. In red are depicted the deleterious SNPs and in blue the protective ones. In grey are highlighted the age of first clinical skeletal muscle symptoms.

Family members have different genomic profile based on WGS study

Among the three family cases identified in this study, members of the same family featuring different clinical severity but similar genomic deletion breakpoint tend to

have a heterogeneous SNPs profile in interrogated genes. Here, we report a family case of three members with two brothers and one nephew featuring different skeletal muscle phenotypes (p8*, p9*, p11*). The main clinical characteristics and genomic characterization are listed in the Table 4.

	Gene	ID	p8*	p9*	p11*
Gene modifiers	<i>TCTEX1D1</i>	rs1060575 rs3816989			
	<i>ACTN3</i>	rs1815739	C I A A M(het)	[Red bar]	[Red bar]
	<i>THBS1</i>	rs1004866 rs2725797 rs2624259 rs814518 rs710160			
	<i>LTBP4</i>	rs2303729 rs1131620 rs1051303 rs10880			
	<i>TGFBR2</i>	rs4522809			
	<i>SPPI</i>	rs28357094			
Clinical status	Age (years)	Actual	14	59	71
		SkM symptoms	9	30	2
SkM deficit		NA	NA	53	
LoA		NA	NA	60	
		Cardiac	normal	normal	CdM
		Pulmonary	normal	normal	normal
		Cognitive	normal	normal	normal
		CSS	MILD	MILD	SEVERE
Dystrophin	Dp427m	WB	uk	60%	uk
	Dp116	WGS	yes	yes	yes
	Dp140	WGS	yes	yes	yes

Table 4 Family case trio of BMD □ 45-55 patient family.

Indeed, as detailed here despite his young age p8* features very mild clinical symptoms with calf hypertrophy and elevated CK. During his last clinical evaluation at 12 years, patient had an excellent clinical status except of a muscle force examination of the upper limb (*triceps brachii*) 4/5 and 5/5 on the rest of his force examination. Gower's sign was negative and no limb contractions were reported. Also, the young boy practice sports with mild limitations and features occasional muscular pain during sustained exercise. His respiratory and cardiac evaluations are normal. His current education is in a regular school and he is one of the best pupils of this class. In addition to his clinical detailed characterization, we searched for his genetic modifiers profile and reported the largest number of protective SNPs which were previously described in literature in DMD patients. Indeed, in comparison to his uncle p9*, this patient was reported to have skeletal muscle symptoms at earlier age (9 y.o.). In contrary, patient p9* featured his first skeletal muscle symptoms at an approximate age of 30 years old, with lower limbs fatigue, calf hypertrophy and quadriceps amyotrophy. Interestingly, the dystrophin protein relative quantification at the diagnostic biopsy was estimated at 60% compared to healthy control. His current cardiac status was not reported recently because of a patient drop-off of the medical follow-up.

The most severe patient, p11* is one of the outliers of this cohort and indeed he doesn't have the same profile of genetic modifiers as his relatives. Indeed, this patient has *ACTN3* and *TGFBR2* SNPs that can be probably associated to the late age of the skeletal muscle deficit. In addition, the lack of the deleterious SNPs confirms the complex mechanism of the skeletal muscle deficit in BMD patients. Unfortunately, we did not have the dystrophin quantification in this patient because the skeletal muscle biopsy wasn't realized at diagnosis.

Altogether this family case reports different genetic modifiers profile in relatives with the same genomic mutation in *DMD* gene. The mild phenotype in this patient trio could be explained by a larger number of protective SNPs. Prospective follow-up of this patients is necessary in order to confirm the reported data.

DISCUSSION

The therapy development in DMD has been largely inspired by the clinical milder phenotype of BMD patients. Several antisense oligonucleotides (AON) treatments are actually FDA and EMA approved and available for DMD patients (EXONDYS 51, VYONDYS 53). Genotype-phenotype studies highlighted the central rod domain sharing deletion of exons 45-55 with less severe muscular involvement, except for rare cases of dilated cardiomyopathy^{17,18,21,35}. Studies with both, antisense oligonucleotides (AON) development and CRISPR/Cas9 systems for NHJR reported encouraging results for the MES45-55 as potential alternative to non-mutation dependent therapies for DMD patients. Here we report the characterization of the largest cohort of BMD patients with specific del45-55 as a predictor to the outcome of MES 45-55 therapy. The clinical characterization of 50 BMD Δ 45-55 patients highlights an exclusively 100% BMD skeletal muscle phenotype. One patient underwent cardiac transplant despite a mild skeletal muscle phenotype. New in-house Clinical Severity Scale (CSS) was assessed retrospectively in 43 patients. For this clinical scale we focused on two major variables responsible for morbidity and mortality: skeletal muscle weakness with the LoA and the presence and severity of cardiomyopathy. As expected, mild and

intermediate type of CSS phenotype are predominant, only one patient was classified as asymptomatic and two patients lost ambulation. The major reported symptoms as fatigue, effort intolerance, high CK levels and transient episodes of rhabdomyolysis are in line with the previously reported studies. Surprisingly, we had only one patient classified as asymptomatic in contrast with the Taglia et al. study²⁰. Interestingly, patients in mild (MBMD) and intermediate (IBMD) are equally represented confirming the progressive character of the muscular dystrophy. Moreover, the patient stratification according to CSS scale allows a better identification of a slowly progressive nature of the muscular dystrophy in this specific exonic deletion. Indeed, both severe and the majority of IBMD all had initially a mild stage evolution of the disease. At ~age of 40 y.o. the patients had a worsening of the clinical phenotype with the installation of skeletal muscle deficit. A prospective follow-up data will be interesting to report for the family case with three brothers who had inaugural skeletal deficit since very early childhood at 4 y.o. revealed that the two SBMD patients had a ~10 years difference between the age of the appearance of skeletal muscle deficit. Despite the retrospective nature of this work, the CSS

classification reveal the slowly progressive character of the muscular dystrophy in BMD patients with the specific exon 45 to 55 deletion.

Here we highlight a high percentage of cardiomyopathy in 33 % of patients with one patient who underwent cardiac transplant. Several studies reported that X-linked cardiomyopathies and BMD share the same distribution of deletion mutation hotspots corresponding to the rod domain (exons 45 to 55). In addition, literature data reported a median age of 43 years as the onset of cardiomyopathy in patients with exon 45-55³⁶. We complete this data and detected a later age of onset at mean 46 years. Surprisingly, despite a later onset the total number of cardiomyopathies was high, at 33 % and represents in our opinion an important issue to be studied in prospective studies. Indeed, as reported in literature, the cardiomyopathies in patients with exon 45 to 55 deletion have a trend to severe complications secondary to viral infections and have a high risk of heart failure³⁷. Despite a general favorable phenotype of this cohort, one French patient reported severe heart failure with rapid onset despite a mild skeletal muscle phenotype that required cardiac transplant. Fortunately, patient recovery is excellent. In previously reported Japanese case report the cardiomyopathy management was also positive, suggesting a high degree of

therapy responsiveness³⁷. Our results underline a significant CSS age-related decline of the left ventricular fraction.

Earlier BMD genotype-phenotype studies revealed that BMD patients with distal rod domain deletions had a classic Becker muscular dystrophy phenotype despite a high clinical and dystrophin protein level variability. Authors reported correlation of the age at reaching the moderate degree with dystrophin levels (relative quantification by WE) in patients with distal rod domain mutations³⁸. This data indicated the functional role of dystrophin domains. Recently, clinical phenotype was correlated with the structural characteristics of dystrophin at the deletion site and found a strong association with phenotype severity in BMD patients. Precisely, modelling study and biochemical data demonstrated that central rod domain deletions can lead to profound structural modifications of dystrophin and influence the disease severity in BMD patients^{26,35}. In this work we looked closely to exon 45-55 specific correlation of the dystrophin levels with the disease progression and we found a negative correlation to the age of diagnosis. In addition, the dystrophin relative quantification from our group and also in several previously published studies underline the highly heterogeneous dystrophin level in patients with the exon 45-55 deletion^{21,35,39-43}. Thus, the present

data indicate that in addition to the structural modification of the truncated resulting protein there are additional factors that are influencing the first skeletal muscle symptoms in Becker muscular dystrophy.

Despite the rare disease character of DMD and a relatively low patient number, genome wide association studies (GWAS) were recently conducted and by two distinct approaches was identified two novel gene modifiers as *TCTEX1D1*, *THBS1*²⁷⁻²⁹. For the *TCTEX1D1* locus deep genotyping of extremely different clinical presentation was validated prospectively in a multi-center European cohort (Bio-NMD) composed of 301 patients with DMD. Whole exome sequencing (WES) performed in DMD patients with extreme phenotypes identified 11 single nucleotide polymorphism (SNPs) associated with age of ambulation loss. Further association of identified SNPs with age of ambulation loss revealed *TCTEX1D1* locus on chromosome 1 influencing the DMD phenotype. Previously, *LTBP4* (IAAM haplotype) was associated with an ~2year delay in time to loss of ambulation in DMD boys^{28,44}. There is no evidence to our knowledge about the presence of these gene modifiers in BMD patients and we explored this issue by conducting a whole genome sequencing (WGS) in 19 BMD Δ 45-55 patients. The WGS data in 19 BMD Δ 45-55 patients were filtered to analyze only variants located in

cardiomyopathy genes (CARDIOGEN list of 65 genes), and this did not lead us to identify any clear SNP potentially associated with cardiac phenotype. Based on these results we report the benign character of the cohort with Mild and Intermediate BMD patients and a limited number of SBMD patients (n=2). In family cases, we did not identify any common variants regardless of the type of analysis. In literature data, patients with early signs of cardiomyopathy and long survivor patients without significant cardiac involvement shared common variants (rs 1060575, rs 3816989). Moreover, the reported variants associated cardiac involvement and loss of ambulation suggesting that identified gene and variants could involve both cardiac and skeletal muscle²⁹. Here we report the absence of any potential pathogenic variants based on our comparative analysis in severe and intermediate patients. However, we cannot exclude that some of the variants classified as variants of unknown significance (ACMG class 3) do not slightly contribute to the cardiac phenotype of the patients⁴⁵. We underline that WGS was conducted in a small cohort and only two patients featured SBMD that could explain the lack of statistical significance in the conducted analysis. The lack of potential candidate involved in cardiac and skeletal muscle phenotype variation in this report cannot exclude their absolute presence but suggest

the importance of better analysis of the regulatory sequences involved in different isoform's expression of *DMD* gene. Another hypothesis is that some modifier variants may be located in genes that are still not studied at the diagnostic level for cardiomyopathies.

Based on the Japanese recent study on Dp116 pathogenic role in cardiomyopathy we searched the WGS data in BMD Δ 45-55 patients in order to identify if the exon 1 of Dp116 localized in intron 55 was conserved on genomic level³⁷. One of the explanations of the cardiac phenotype variability could be the promoter activation of the Dp116 localized in the intron 55. Indeed, in Yamamoto et al revealed by PCR amplification of Dp116 transcript promoter activation in human cardiac muscle and surprisingly an association for the Dp116 with cardiomyopathy in DMD. Surprisingly, the shorter isophorme of dystrophin as Dp116 did not act as expected the protective compensatory role but in contrary was pathogenic. The Dp116 isophorme was originally described in peripheral nerves and brain and this recent data add a new potential actor in phenotype variability in BMD patients. Indeed, from 4 candidates with cardiomyopathy in BMD Δ 45-55 cohort only two of them preserved the exon 1 of Dp116 isophorme. Moreover, the severe patients had different genetic pattern, patient N11 conserved the

exon 1 of Dp116 isophorme and patient 19 did not. Interestingly, both patients lost ambulation and despite the onset of first symptoms in early infancy, patient N11 had a rapid decline of skeletal muscle deficit compared to patient 19. Unfortunately, we did not had access to muscle biopsy in order to confirm the expression of Dp116 isoform is these two patients.

As mentioned, literature data reported several genetic modifiers involved in phenotype variability in DMD patients and we hypothesized that same SNPs in genetic modifiers could be involved in phenotype variability in BMD patients. Indeed, BMD patients have generally a milder phenotype and a present but truncated dystrophin protein. We took advantage of this particular feature and analyzed BMD patients with same exonic deletion from 45 to 55. Deep phenotype characterization allowed to identify phenotype variability of skeletal muscle features and assessment of the disease progression by a clinical severity scale.

The majority of reported SNPs are involved in major canonic pathways of great interest in DMD/BMD environment. Indeed, *SPP1*, *TGFBR2*, *LTBP4*, *THBS1* are involved in TGF- β pathway^{28,30,32} and *TCTEXD2* has a potential link to SMAD pathway²⁹. As per *ACTN3* is of a great importance in skeletal muscle structure especially in fast fibers⁴⁶.

LTBP4 regulates the transforming growth factor (TGF- β) and several SNPs result in two major protein coding haplotypes, one coding for a protective protein isoform (IAAM) and the second one associated with a deleterious protein isoform. *LTBP4* SNPs haplotypes display the protective effects with a recessive model. Among several reported SNPs, rs710160 alleles are differentially distributed between VTTT/IAAM haplotypes and phased analysis from DMD patients identified that the rs710160_C allele sub-divided the IAAM haplotype. Despite a largely favorable phenotype of the cohort reported here, there is only one patient with the protective haplotype C-IAAM from 19 screened BMD Δ 45-55 patients. In addition, the same patient has protective SNPs in *ACTN3* and *TGFBR2* featuring the larger number of SNPs.

However, in this study we reported 3 patients with *LTBP4* SNPs that are resulting in a potential deleterious isoform. In two of these patients (p31 and p21) the age at first clinical signs was in the early childhood. Interestingly, p21 has an association with a protective *ACTN3* SNP and despite an advanced actual age this patient is still ambulant. In contrary, p31 doesn't have any association with other protective SNPs, taking into consideration his young age, 20 years old, a prospective follow-up might be necessary in order to confirm his skeletal muscle evolution.

Another important partner of the TGF β signaling pathways is the thrombospondin-1 (TSP-1 is encoded by *THBS1* gene) that interacts with *LTBP4*²⁷. The rs2725797 associated with LOA and interestingly in skeletal muscle it was reported that rs2725797 and rs rs710160_C result in average with 6.8 years delay of LoA. We supposed that this cumulative feature will be similar for the SNPs described in *ACTN3* gene. Indeed, the α -actinine-3 deficiency due to a null mutation (R577X) in *ACTN3* gene results in a protective oxidative muscle metabolism with age. Literature data reported that oxidative fibers are relatively spared from degeneration in DMD⁴⁶. In addition, *ACTN3* genotype significantly influence elite athletes and healthy individuals. Here, we report rs1815739 as the most frequent SNPs in this BMD specific cohort.

Recent WES study identified *TCTEXD2* SNP associated with the LoA in DMD patients²⁹. In this cohort we report one patient featuring both reported SNPs but we did not find a positive association with the LoA as the patient remain ambulant till late age. We detailed a family case (p8*, p9*, p11*) that illustrated the importance of the WGS in patient stratification. Also, this detailed genomic characterization can allow a better prognosis of disease long term evolution if the diagnosis is performed early in a positive family history. Prospective

follow-up will be necessary in order to confirm the potential involvement of the previously described gene modifiers in severe DMD patients to the benign BMD Δ 45-55 cohort.

These results suggest that genetic modifiers have still unrevealed important phenotype regulator partners as might be the non-coding RNA. For example, lncRNA H19 was recently reported to alleviate DMD phenotype by stabilizing dystrophin⁴⁷.

In conclusion, this study confirms the importance of the genetic modifiers profile in disease evolution prognostic in a personalized medicine approach.

Furthermore, new regulatory non-coding sequences were described as important regulatory elements in myogenesis. A variety of lncRNA specific to *DMD* was described by Bovolenta et al. in Duchene female carriers⁴⁸. In fact, their forced expression in human muscle and neuronal cell caused a negative regulation of

endogenous full-length dystrophin isoforms (brain, muscle and Purkinje). The authors hypothesize that the mechanism of action involves specific DMD lncRNA that control muscle dystrophin isoforms by down-modulating dystrophin transcription levels. Unfortunately, in this study there is no correlation data with a more severe phenotype as limited to DMD female carriers. From this report, four lncRNA draw our attention as their localization is boarding the exon 45 and 55 and thus becoming an interesting target of further analysis.

In conclusion, the genotype characterization established its utility in future work to identify benign phenotypes of BMD patients that could be beneficial for the therapy design. This study will shed new light onto the genotype-phenotype link in specific BMD del45-55 cohort, thus increasing the ability to predict the outcome of MES strategy therapy.

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ARTICLE 2

Context:

We defined as Aim 2 the identification of new actors of phenotype variability and dystrophin regulators. In line with this objective we report in article N2 entitled: “Applicability of the lncRNA44s2 study to the design of 45-55 exon skipping tool for DMD therapeutic strategy” the results of lncRNA boarding exon deletion 45-55 characterization.

Abstract:

Recent data showed that long ncRNAs (lncRNA) might be important regulators of proliferation and differentiation processes in skeletal muscle, thus representing promising therapeutic targets and/or biomarkers for Duchenne and Becker muscular dystrophy (DMD/BMD). DMD and BMD are X-linked myopathies characterized by a progressive muscular dystrophy with or without dilatative cardiomyopathy. Two third of *DMD* gene mutations are represented by deletions and 63% of DMD patients are eligible to 45 to 55 multi-exons skipping (MES), thus becoming BMD patients (BMD Δ 45-55)¹⁹⁰. We previously reported clinical variability in the largest BMD Δ 45-55 cohort. We highlighted that all four lncRNA are differentially expressed during myogenesis in immortalized and primary human myoblasts. In addition, the lncRNA44s2 was pointed out as an accelerator of differentiation. Interestingly, lncRNA44s was associated with a favorable clinical phenotype.

This work is under preparation for publication submission for the Special Issue in “Non-coding RNAs in Health and Disease” of Biomedicines journal.

Applicability of the lncRNA 44s2 study to the design of 45-55 exon skipping tool for DMD therapeutic strategy

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ABSTRACT

Background: Noncoding RNAs (ncRNAs) have a major role in the regulation of gene expression in physiological and various pathological conditions. Recent data showed that long ncRNAs (lncRNA) might be important regulators of proliferation and differentiation processes in skeletal muscle, thus representing promising therapeutic targets and/or biomarkers for Duchenne and Becker muscular dystrophy (DMD/BMD). DMD and BMD are X-linked myopathies characterized by a progressive muscular dystrophy with or without dilatative cardiomyopathy. Two third of *DMD* gene mutations are represented by deletions and 63% of DMD patients are eligible to 45 to 55 multi-exons skipping (MES), thus becoming BMD patients (BMD Δ 45-55)¹. We previously reported clinical variability in the largest BMD Δ 45-55 cohort.

Objective: In order to decipher the phenotype variability, we analyzed the genomic lncRNA profile in 38 BMD Δ 45-55 patients and characterized four of lncRNA localized in introns 44 and 56 of *DMD* gene.

Results: We highlighted that all four lncRNA are differentially expressed during myogenesis in immortalized and primary human myoblasts. In addition, the lncRNA44s2 was pointed out as an accelerator of differentiation. Interestingly, lncRNA44s expression was associated with a favorable clinical phenotype.

Conclusion: These findings suggest that lncRNA44s2 is involved in muscle differentiation process and could be a potential disease progression biomarker. Based on these results, we support the MES45-55 therapy and propose that the design of CRISPR/Cas9 MES45-55 assay considers the lncRNA sequences boarding the exonic 45 to 55 deletion.

Key words: long non-coding RNA, lncRNA, ncRNA, Becker muscular dystrophy, BMD, Duchenne muscular dystrophy DMD

INTRODUCTION

Skeletal muscle is the principal actor of the human body movement and posture maintenance. The genesis of the skeletal

muscle -myogenesis- encounters several distinct phases^{2,3}. Each step of myogenesis is orchestrated by complex intrinsic and

extrinsic regulators including the myogenic regulatory factors (MRF), a group of highly conserved genes expressed in skeletal muscle lineage as myogenic factor -*Myf5*, myogenic differentiation antigen-*MyoD*, myogenin-*MyoG*, myogenic regulatory factor- *MRF-4*, myosin heavy chain-*MHC*^{4,5}. The MRF altogether with the Paired box (Pax) family members Pax3 and Pax7 act at hierarchically defined timepoints to regulate cell cycle, proliferation and differentiation^{6,7}. Moreover, literature data underline that common progenitor cell maintains muscle growth during late embryonic development and assure the adult muscle growth and repair⁷⁻⁹.

Along with the multiple myogenic regulatory factors, the long non-coding RNA (lncRNA), a potential, emerge as new key role players¹⁰⁻¹³. Aberrant regulations of growth and the development process of skeletal muscle acts as an important step in the pathophysiology of various neuromuscular disorders. Recent high-throughput RNA sequencing (RNAseq) experiments identified lncRNA that are differentially expressed during skeletal myogenesis^{14,15}. In addition, some of these transcripts are dysregulated in dystrophic versus wild-type muscles suggesting an involvement in the pathological course. Alterations in the regenerative and dystrophic processes may influence the disease

progression and patient outcome including in Becker and Duchenne muscular dystrophies (BMD/DMD).

Both BMD/DMD are X-linked allelic disorders caused by mutations in *DMD* gene^{16,17}. DMD is characterized by mutations leading mRNA transcription interruption resulting in absent dystrophin protein and severe muscular deficit with loss of ambulation (LoA) in young boys and shortened life span. In contrast, during the BMD pathophysiologic process the mRNA open reading frame is maintained resulting in the production of a shortened but functional dystrophin leading to a less severe phenotype. Due to this exceptional particularity the therapeutic dogma is to convert DMD into BMD by altering the pre-mRNA splicing by exon skipping in order to restore the open reading frame allowing the translation of an internally deleted and partially functional protein¹⁸⁻²⁰. Actual FDA/EMA approved antisens oligonucleotides (AONs) as EXONDYS 51(exon skipping 51) and VYONDYS 53 (exon skipping 53) cover up approximative 10% and 13 % of patient deletion mutation respectively. In order to enlarge the coverage of patient number eligibility up to 63% of patient deletion mutation, the multiple exon skipping (MES) of exons 45 to 55 by antisens oligonucleotides

(AON) and CRISPR-Cas9 therapies are currently in development. Thereby, the nonhomologous end-joinment (NHEJ) reframing of *DMD* 45-55 exons in immortalized DMD patient muscle cells (ex.48-50del) lead to successful dystrophin rescue²¹⁻²³.

In our previous study (Gargaun et al. in preparation), we have underlined the phenotype variability in Becker patients with exon 45-55 deletion (BMD Δ 45-55). Here we report breakpoint analysis by whole genome sequencing (WGS), performed in 19 patients of the BMD Δ 45-55 population and show that the specific breakpoint involves the deletion of the sequences of lncRNA. To go further, we analyzed the presence of lncRNA boarding the *DMD* 45-55 deletion in 38 BMD Δ 45-55 patients and examined their skeletal muscle expression. The lncRNA 44s and 44s2 expression pattern was studied in skeletal muscle biopsy of control, BMD Δ 45-55 patient, BMDdel3-7 patients and BMD Δ 45-55. Profile of expression of these lncRNA in dystrophic immortalized and primary cells (Myo45-55/Myo45-52) confirmed what was observed the skeletal muscle biopsies. Furthermore, overexpression of the ncRNA44s2 revealed its role in skeletal muscle regeneration mechanism.

Altogether, these results raise the question of the correlation between the favorable clinical phenotype of BMD Δ 45-55 and the lncRNAs in intron 44.

MATERIALS AND METHODS

Ethic statement

This study was reviewed and approved by the French Institutional Review Board. Written consent was obtained for the diagnostic purposes. All participants were assigned de-identified numbers. Diagnosis muscle biopsies were collected from BMD and DMD patients and healthy controls with signed informed consent.

Cell culture

Isolated human primary myoblasts from healthy subject and DMD 45-52 deletion patient were kindly provided by Pr F Muntoni (King's College London, UK). The BMD Δ 45-55 human primary myoblasts were provided by the Dr Streichenberger (HCL Lyon, France). The human immortalized lines (Myo45-52 and WT) were provided by Dr V Mouly, Dr K Mamchaoui and Dr A Bigot (MyoLine facilities Institute of Myology, Paris, France) (Mamchaoui et al, 2011).

For proliferation, both primary and immortalized myoblasts were maintained in skeletal muscle cell growth medium (DMEM supplemented with 5µg/ml of insulin, 5ng/ml of EGF, 0.5ng/ml of bFGF, 0.2 µg/ml of dexamethasone only for immortalized cell lines, 25µg/ml of fetuin, 20% of fetal bovine serum and 16% of medium 199). For differentiation experiments, primary and immortalized myoblasts were maintained in DMEM supplemented with 2% horse serum. Proliferation and differentiation for both primary and immortalized cell lines were supplemented with 50ug/mL of gentamycin.

DNA/RNA purification and analysis

Healthy control and BMD, DMD patient's DNA extraction from diagnostic blood samples were analyzed by DNA kit (Macherey-Nagel). Total RNA of skeletal muscle biopsies and human primary and immortalized myoblasts from a healthy subject (ctrl) and from a DMDdel45-52 patient (DMDdel45-52) and BMDΔ45-55 patient (DMDdel45-55) extractions were performed using Nucleospin miRNA kit (Macherey-Nagel). Reverse transcription (RT) was performed on 250 ng of mRNA by using the Superscript II and random hexamers (Life Technologies). RT-PCR products were separated by electrophoresis on 3% Agarosis

gel with ethidium bromide. qPCR was performed on OneStep system with SYBR Green/ TaqMan reagent using primers shown in Table 1. Relative expression between proliferating and differentiated myoblasts and transduced and control myoblasts was measured in duplicates with in 4 separate experiences. Statistical significance was analyzed using two-tailed Student's test and ANOVA on GraphPad Prism.

Adeno-associated virus vector (AAV) production and lncRNA overexpression experiments

The lncRNA44s2 cDNA sequence has been cloned in a pSMD2 plasmid (lnc44s2-pSMD2) under the control of the strong CMV promoter and a polyA signal (Addgene pSMD2). After the bacterial colony and plasmid amplification, the lnc44s2-pSMD2 was transmitted to the AAV facility (Center of Research, Institute of Myology) for the generation of AAV9- lnc44s2 Serotype 9 AAV vectors were generated using a three-plasmid transfection protocol as described elsewhere(ref). The proliferation and differentiation medium were specified earlier.

For the proliferation experiments, the human immortalized and primary myoblasts from control, BMD Δ 45-55 and DMD45-52 patients were plated 24 h before transduction (D0) ($3.5E+05$) in a 24-well plate in proliferation medium. At D1 myoblasts were transduced at MOI ($3.0E5$) in 300 μ l with AAV-nc44s2, AAV-Mock-GFP and transduction medium for the wild type (WT) condition. Cells were counted on the Acova plate and the cell platelet was collected at the third day of proliferation (P3).

For the differentiation experiments, the human immortalized and primary myoblasts from control, BMD Δ 45-55 and DMD45-52 patients were plated 24 h before transduction (D0) ($5.0E+05$) in a 24-well plate until confluent (approximative at 80% of cell confluence) and then switched to differentiation medium. At 80% of confluence the myoblasts were transduced at MOI ($3.0E5$) in 300 μ l with AAV-nc44s2, AAV-Mock-GFP. In wild type (WT) condition the proliferating medium was changed to differentiation medium at 80% of confluence. Cell platelet was collected at the third day of differentiation (D3).

Immunohistochemistry

Primary and immortalized human myotubes were fixed with Paraformaldehyde 4% 10

min at RT and washed 3 times in PBS. Fixed cells were permeabilized with 0.5% Triton X-100/PBS (Sigma-Aldrich), for 10 min at room temperature and blocked with bovine serum albumin BSA 4% for 45 min at room temperature. Primary hybridoma antibody MF20 (Institute of Myology) were applied overnight at 4°C, washed and visualized with fluorochrome-conjugated secondary antibody Donkey anti-goat (Alexafluor 594 conjugate, Life Technologies, 1:500) for 1h at room temperature. Fixed cells were washed in PBS/1% BSA/0.1% saponin and then in PBS for 10 min. Images were analyzed with EVOS microscope using x 10/x20 objective.

Bioinformatic analysis

Whole Genome Sequencing (WGS) was performed in 19 patients by Illumina sequencing. The bioinformatic analysis of each patient in this group was performed by two different bioinformatic tools: VarAFT Annotation and Filter for the variant analyses. VarAFT provided experiments quality, annotation and filtration of VCF files²⁴. The DNA breakpoint analysis, was performed by the BreakDancerMax tool that could predict five types of structural variants: insertions, deletions, inversions, inter- and intra-chromosomal translocations. This method allows to treat next-generation short paired-

end sequencing reads using read pairs that are mapped with unexpected separation distances

RESULTS

BMD Δ 45-55 patients lncRNA breakpoints impact in lncRNA sequences

In our previous work we underlined a clinical phenotype variability in the largest cohort of Becker patients (BMD Δ 45-55) and demonstrated the involvement of the miR-708 and miR-34c in the regulation of the nNOS expression and therefore to the pathomechanism of dystrophic muscle of these BMD patients²⁵. The present research raised the question of the regulation

or orientation. The deletion read precision is about +/- 20 base pairs.

mechanisms in the phenotype variability in BMD Δ 45-55 patients. To determine the precise deletion coordinates a Whole Genome sequencing (WGS) analysis was carried out in 19 patients. This analysis was realized using Breakdance software and we have achieved a +/- 20 pair base precision deletion coordinates (Figure 1) and based on WGS determined that each patient displayed a specific deletion breakpoint. We then investigated the impact of the specific breakpoint on the lncRNA sequence presence in the WGS analyzed patients (Table 1).

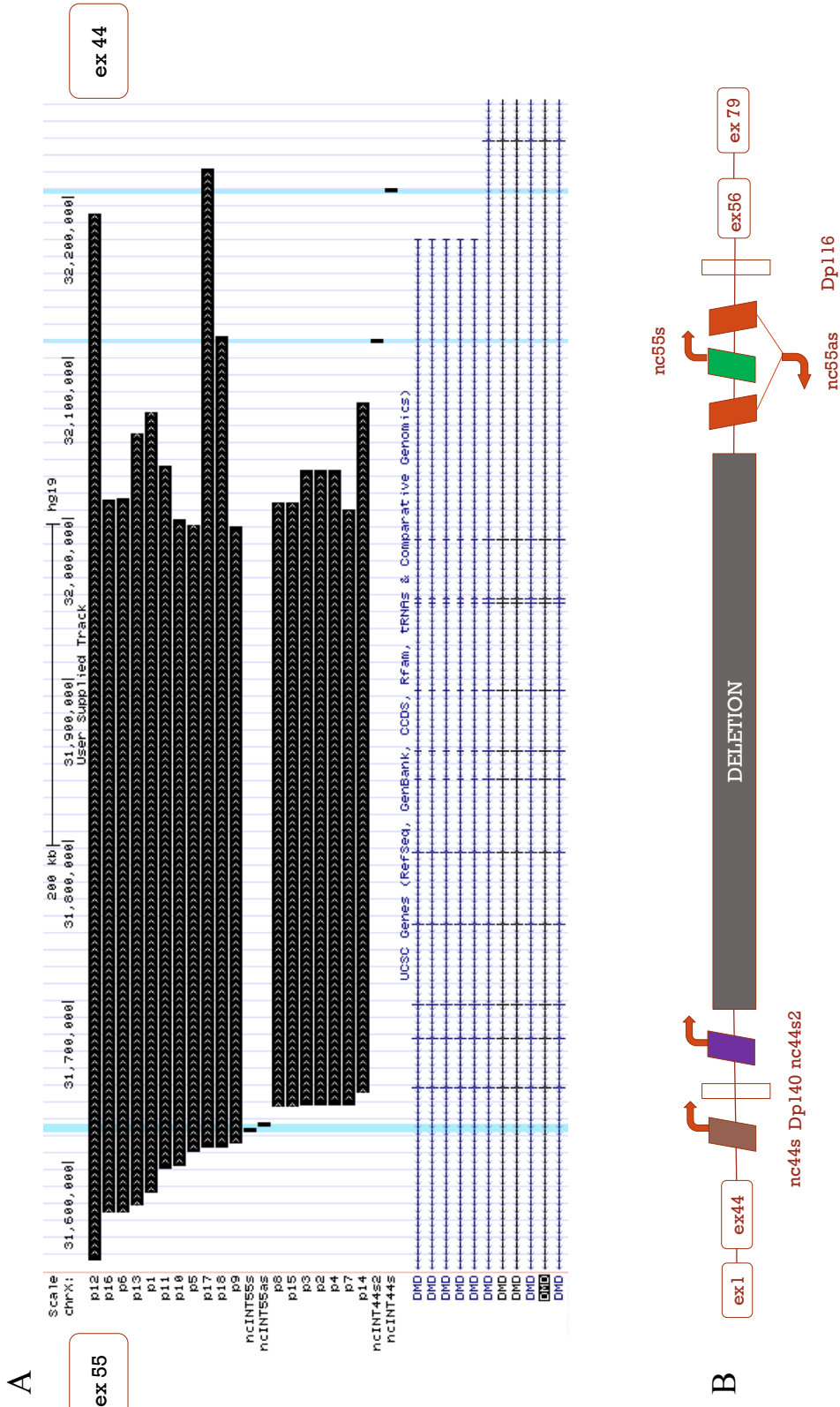


Figure 1 Neo-intron of deleted 45-55 exons in DMD gene. (A) Scheme presentation of the WGS detection of the deletion breakpoint (black boxes) in 19 patients BMD Δ 45-55. (B) Scheme presentation of the canonic exon 45-55 deletion neo-intron with the lncRNA (colored boxes for lncRNA and arrows to indicate the transcription sense). The exons 1 to 44 and 56 to 79 are illustrated in clear red boxes).

BMDdel45-55 patients N°	lncRNA genomic sequence					DNA profile
	44s	44s2	55s	55as	no lncRNA	
1	1	1	0	0	0	PPNN
2	1	1	0	0	0	PPNN
3	1	1	1	1	0	PPPP
4	1	1	1	1	0	PPPP
5	1	1	0	0	0	PPNN
6	1	1	0	0	0	PPNN
7	1	1	1	1	0	PPPP
8	1	1	1	1	0	PPPP
9	1	1	1	1	0	PPPP
10	1	1	0	0	0	PPNN
11	1	0	0	0	0	PNNN
12	1	1	1	1	0	PPPP
13	1	1	0	0	0	PPNN
14	1	1	0	0	0	PPNN
15	1	1	0	0	0	PPNN
16	1	1	0	0	0	PPNN
17	1	0	0	0	0	PNNN
18	1	0	0	0	0	PNNN
19	1	1	0	0	0	PPNN
20	1	0	0	0	0	PNNN
21	1	0	0	0	0	PNNN
22	1	1	1	1	0	PPPP
23	1	1	1	1	0	PPPP
24	1	1	1	1	0	PPPP
25	1	0	0	0	0	PNNN
26	1	1	1	0	0	PPPN
27	1	1	1	1	0	PPPP
28	1	1	1	1	0	PPPP
29	1	1	0	0	0	PPNN
30	1	1	0	0	0	PPNN
31	1	1	0	0	0	PPNN
32	1	1	0	0	0	PPNN
33	1	1	0	0	0	PPNN
34	1	1	0	0	0	PPNN
35	1	1	0	0	0	PPNN
36	1	1	0	0	0	PPNN
37	1	0	0	0	0	PNNN
38	0	0	0	0	1	NNNN

Table 1 lncRNA genomic sequence presence detected by PCR in 38 patients.

Only one BMD Δ 45-55 patient displayed a large deletion that involved all lncRNA sequences (2%), in 7 patients the lncRNA 44s2,55s and 55as were deleted (18%), in 18 patients the deletion breakpoint removed only

the lncRNA localized in intron 55 (47%) and in 11 patients (29%) the deletion conserved all the lncRNAs. Overall, we report a major trend of intron 44lncRNA conservation and a second one conserving all lncRNAs. To

further increase the significance of this observation; the breakpoint profiling was completed by the detection of lncRNA sequences boarding the exonic deletion in a total of 38 patients (19 additional BMDdel45-55 patients) on DNA samples extracted from diagnostic blood samples (Table 1). The results revealed two most frequent deletion breakpoints, one is preserving the non-coding sequences localized in the intron 44 and the second one is conserving all the lncRNA both

from intron 44 and intron 56. These data highlighted that the lncRNA 44s and 44s2 are spared by the deletion in the majority of the BMD Δ 45-55 patients (n=30, 79%)(Figure2B). Also, despite the increase in the patients number only in one patient all lncRNA sequences were deleted. Analysis of available features of clinical phenotype revealed the trend to less severe clinical phenotype in patients with the conserved lncRNA44s (Figure2C).

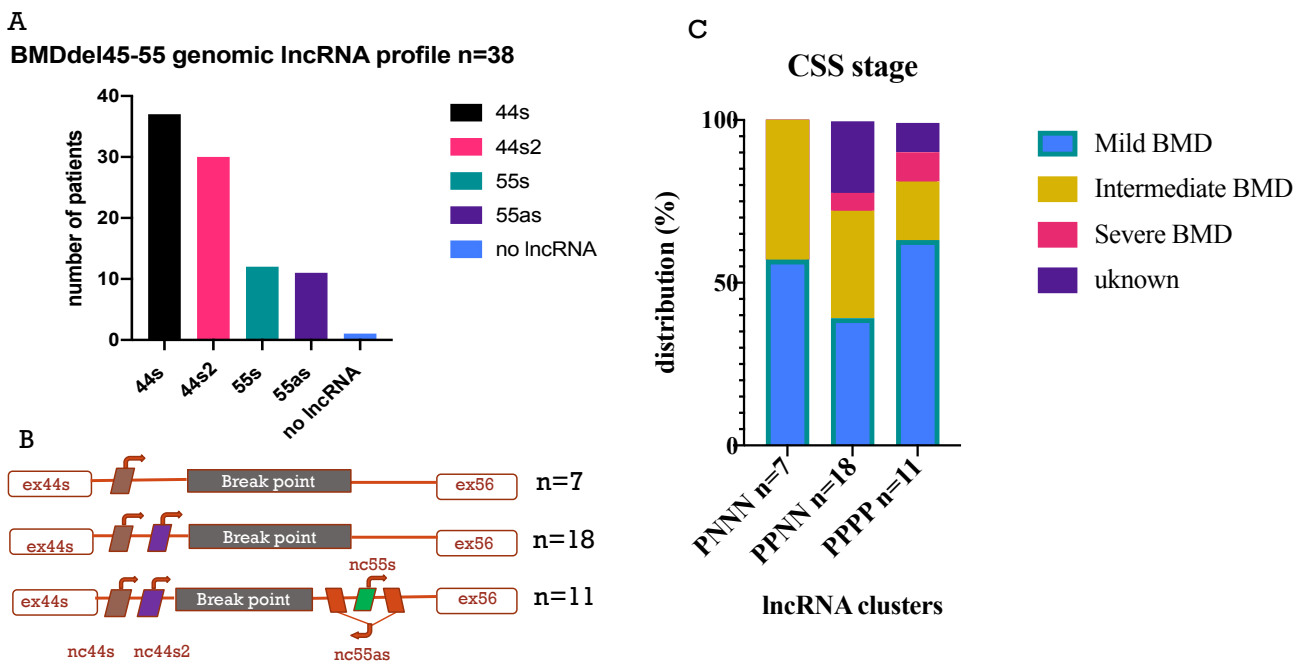


Figure 2 lncRNA profile in BMD Δ 45-55 patients (n=38). (A) lncRNA detection by PCR from diagnostic DNA. (B) Scheme presentation of the most frequent lncRNA genomic presence profiles (C) Phenotype correlation with lncRNA(nc) genomic clusters Abbreviations: MBMD =Mild, IBMD=Intermediate, SBMD= Severe Becker muscular dystrophy, ex=exon)

lncRNA expression BMD/DMD patients
skeletal muscle biopsies

lncRNA expression was investigated in skeletal muscle biopsies of 4 BMD (BMD5, 15, 6, 29, 23), 4 DMD patients (del45-50x2, del51, del51-55) and in 3 controls by RT-PCR. Data showed that the lncRNA 44s2 and 55s were poorly expressed in the patient DMDdel51-55 compared to the control, DMDdel51 and DMDdel45-50. (Figure 3). In addition, the lncRNA 55as expression was not detectable in all tested DMD patients and its expression was highly variable in two of the controls (Figure 3 A).

To explore the issue of the lncRNA expression variability a relative quantification by RT-qPCR was performed in additional muscular biopsies :3 BMD Δ 45-55, two BMDdel3-7, one BMDdel3-5 patient and 3 DMD patients (DMD8=del17-44, DMD14=del46-52, DMD7=del48-52) (Figure 3 B). Indeed, to address the question of the potential impact in the spectrin repeat deleted region on the expression of the lncRNA, we selected several BMD patients with different in-frame deletion mutations.

For the DMD patients, the choice of the mutation was influenced by the available muscular biopsies and also by the exon skipping eligibility of the mutations, patient DMDdel7-44 theoretically would be eligible to exon 45 skipping, patient DMDdel46-52 to MES 45-55 and patient DMDdel48-52 to exon skipping 53.

First, the lncRNA expression analysis showed that all the lncRNA were expressed in all control subjects' biopsies. Secondly, the BMD Δ 45-55 patients had a similar expression level of all lncRNA compared to controls. Interestingly, all BMD Δ 45-55 patients lncRNA expression levels was higher compared to the lncRNA expression in BMDde3-5/3-7 patients. Finally, the lncRNA expression in DMD patients was significantly lower compared to control and BMD Δ 45-55 patients. Altogether, these data underlined a lower expression of lncRNA localized in the intron 44 and 56 in DMD patients. However, in BMD Δ 45-55 patients' biopsies similar expression to control and statistically significantly higher expression compared to BMDdel3-7 patients was observed.

Subjects	Genomic status	lncRNA profile
Control	Healthy controls	PPPP
BMDdel45-55	3 patients del45-55	PPNN
DMD	DMD7-44, DMD46-52, DMD48-52	PPPP
BMD	BMDdel3-7 (n=2), BMDdel3-5 (n=1)	PPPP

Table 2 Genomic profile of control, DMD/BMD patient selected for the expression analysis in muscular biopsies.

lncRNA profile in controls and DMD/BMD patients

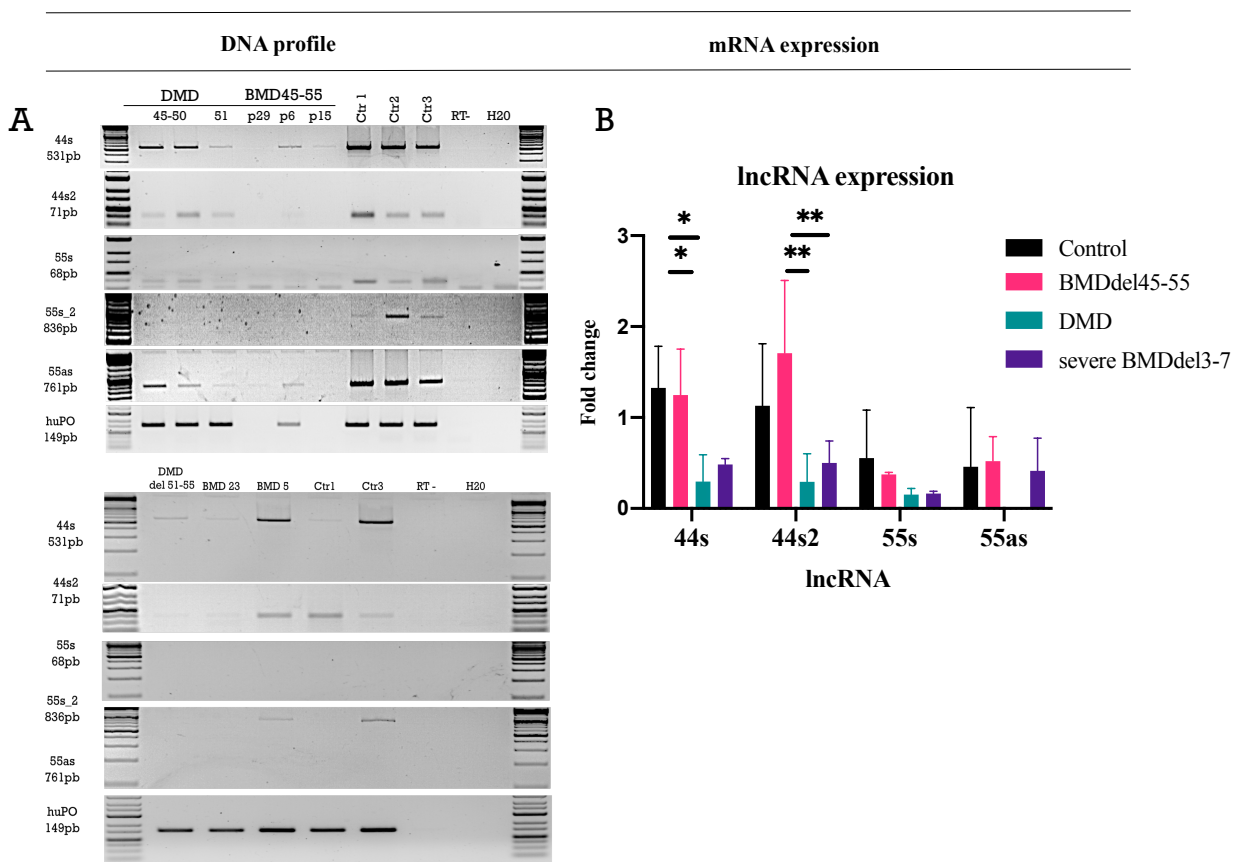


Figure 3 lncRNA profile in control and DMD/BMD patients. (A) lncRNA expression assessment by RT-PCR of 4 lncRNA (nc55s2 assessment was designed because of alternative splicing feature of 55s lncRNA). (B) expression assessment of lncRNA in muscular biopsies of control, DMD, BMD patients by RT-qPCR Abbreviations: ctr=control, DMD=Duchenne muscular dystrophy, BMD=Becker muscular dystrophy

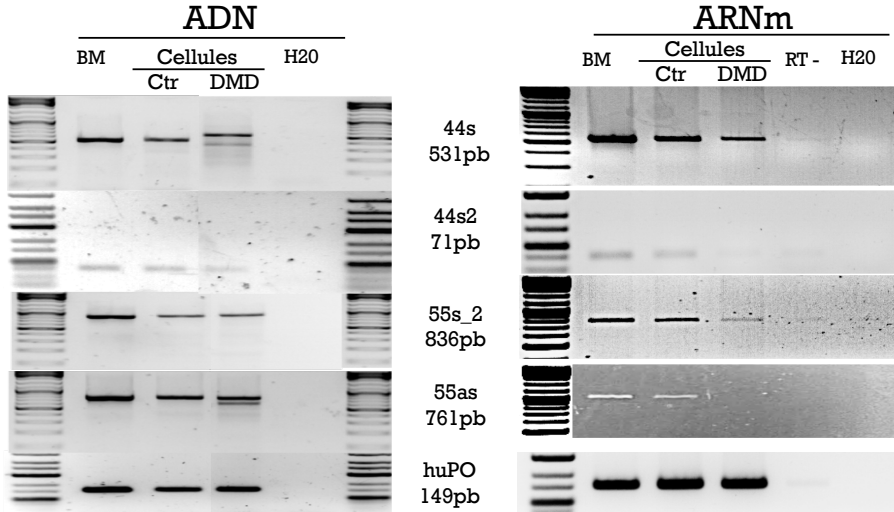
Low lncRNA expression during proliferation in dystrophic human myoblasts

lncRNA expression analysis in patient muscular biopsies pointed out the lncRNA44s2 was less expressed in patients with severe dystrophic condition. Therefore, to address the potential involvement of the lncRNA44s2 in the dystrophic process, we took advantage on having immortalized cell lines from healthy subject (WT-I) and DMDdel45-52 (Myo45-52-I) patients. First, presence of the lncRNA sequences was investigated at the genomic level in these two cell lines and in a muscular biopsy (healthy control). All of the lncRNA sequences were found complete in DNA of both, cellular lines and muscular biopsies. Expression of these lncRNA was then investigated by RT-PCR.

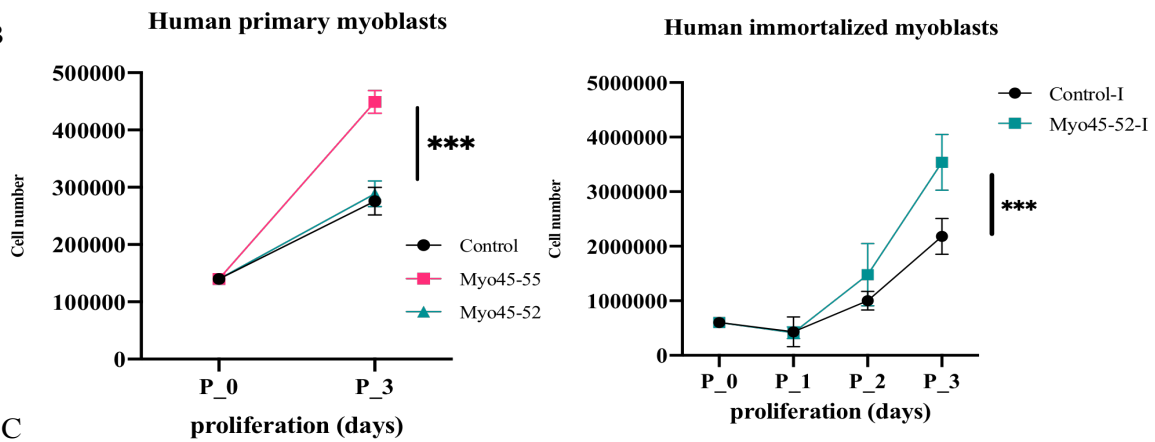
Data revealed the expression of all lncRNAs in WT-I myoblasts and the missing lncRNA44s2 and 55as expression in Myo45-52-I cells (Figure 4A).

The profile of the lncRNA expression was then addressed during the proliferation process. First, a comparative analysis of the proliferation rate of the two immortalized cell lines was performed and showed a statistically significant higher rate of Myo45-52-I cells proliferation compared to WT-I myoblasts (Figure 4B). Then, lncRNA expression was investigated during proliferation and the results revealed a significantly weaker expression of all lncRNA in Myo45-52-I compared to control WT-I myoblasts (Figure 4C right).

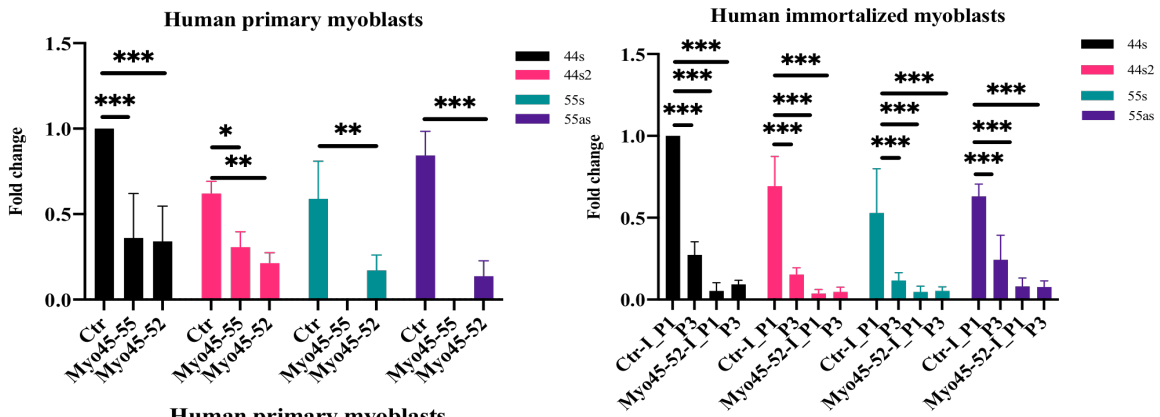
A



B



C



D

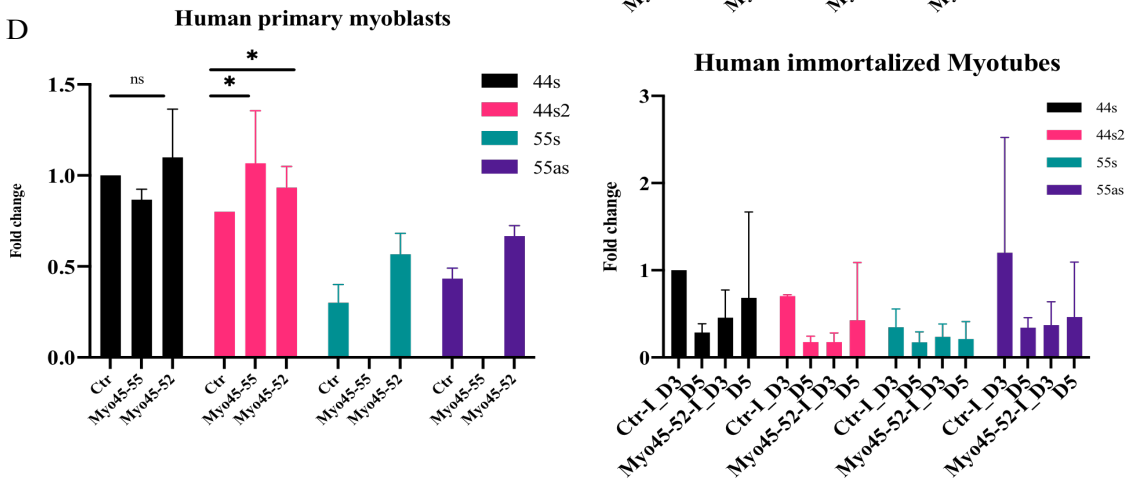


Figure 4 lncRNA genomic profile and expression assessment in human myoblasts. (A) DNA and RNA profiling in muscular biopsy from healthy control (ctr) and immortalized human myoblasts from healthy control and DMDdel45-52 deletion patient (Myo45-52-I). (B) Proliferation assessment at different timelines (day 0-to day 3/P0-P3) in human immortalized and primary human myoblasts from healthy control, BMDΔ45-55 (Myo45-55) and DMD45-52 patients. Proliferative rate is illustrated in a growth chart for each cell line. (C) lncRNA expression profile during proliferation (left) in human primary and (right) in immortalized myoblasts (D) lncRNA expression profile during differentiation (left) in human primary and (right) in immortalized myoblasts (black columns illustrate nc44s, red columns=nc44s2, green columns=nc55s, violet columns=nc55as) were analyzed by RT-qPCR.

In addition, the genomic lncRNA sequences were investigated in primary human myoblasts of control, BMDΔ45-55(Myo45-55) and DMDdel45-52(Myo45-52) patients. First, proliferation rate was shown to be significantly higher in Myo45-55 cells compared to WT and Myo45-52 (Figure 4B). Consequently, during proliferation the expression profile in Myo45-55 and Myo45-52 of all lncRNA of interest displayed a significantly lower expression compared to control myoblasts (Figure 4C left). Interestingly there is no difference between the lncRNA 44s/44s2 expression level between the Myo45-52 and Myo45-55 at day 3 of proliferation (Figure 4C left).

In conclusion, lncRNA expression analysis at three days of proliferation in both, immortalized and primary cell lines identified a significantly lower level of expression in dystrophic cell lines compared to control. Proliferation rate experiments identified higher rate of proliferation in dystrophic myoblasts in both cell lines. Compared to control, Myo45-55 myoblasts displayed the highest rate of proliferation in primary cell

lines and Myo45-52 in immortalized cell lines.

Low lncRNA expression during differentiation in human myoblasts

The differentiation capacity of the myoblasts was evaluated by the fusion index calculated at 3 days of differentiation (myotubes nuclei count/total nuclei number in at least three representative images). This index revealed significantly lower differentiation of Myo45-52-I compared to WT-I myoblasts. As expected, Myo45-52-I illustrated a lower number of myotubes compared to WT-I immortalized myoblasts. In primary myoblasts, the fusion index in Myo45-55 showed significantly higher differentiation compared to WT (fusion index = 0.6) and Myo45-52 (fusion index = 0.3) (Figure 4C).

The lncRNA expression was investigated at 3 days of differentiation in immortalized myoblasts and showed a trend to higher expression in WT-I compared to Myo45-52-I cells but we could not demonstrate a significant difference between the conditions due to high cellular variability (Figure 4D

right). Furthermore, at 3 days of differentiation the expression of lncRNA 44s in Myo45-55 and Myo45-52 reached a similar level of fold change expression to respectively). The nc44s and 44s2 have approximately 4 times higher expression compared to proliferation assessment (Figure 4D left). lncRNA 55s and 55as were absent in Myo45-55 during proliferation and differentiation assessments reflecting the genomic deletion pattern.

Based on the results of lncRNA expression investigations in skeletal muscle biopsies and according to the expression profile of the lncRNA in human myoblasts, the lncRNA 44s2 was selected as candidate for the lncRNA mechanism study during myogenesis.

Interestingly, as depicted in Figure 5, we undergone nc44s2 Over Expression (OE) experiment in the same cell lines. At 3 days of proliferation, in Myo45-52 significantly lower dystrophin mRNA expression was reported between OE nc44s2 condition (AAV9nc44s2) and control. Surprisingly the dystrophin mRNA expression in Myo45-52

control (fold change at 1.2) and for Myo45-55 and Myo45-52 reached a significantly higher level than control (1.2 and 1.0

lncRNA from intron44 and 55 were described as downregulating the full-length mRNA dystrophin expression in in vitro experiments and DMD female carriers (Bovolenta et al). Thus, we analyzed the mRNA level of dystrophin expression by RT-qPCR, during proliferation and differentiation. DMD myoblasts do not express dystrophin protein and already at 3 days of differentiation Myo45-52 and Myo45-55 human primary myoblasts expressed significantly lower dystrophin transcripts compared to WT.

was higher compared to control condition. However, similar trend was observed at 3 days of differentiation in Myo45-52 condition and control myoblasts. From reported results we could not confirm the downregulation of dystrophin mRNA at three days of differentiation (Figure 5).

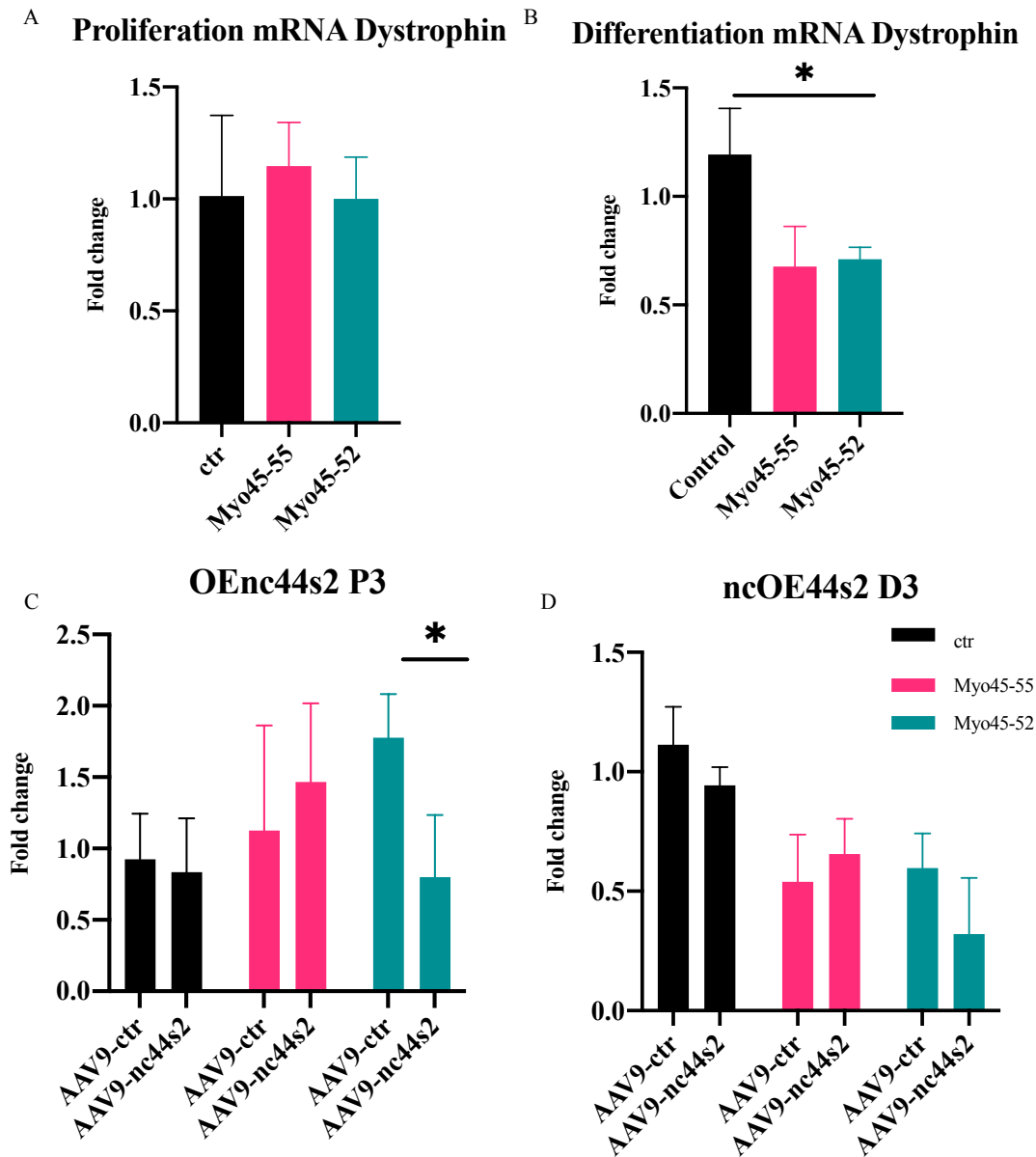


Figure 5 mRNA dystrophin expression assessment. (A) mRNA dystrophin expression assessment by RT-qPCR Taqman at three days of proliferation reveals similar level of expression in human primary myoblasts (ctr, Myo45-55, Myo45-52). As expected, we reveal significant difference of mRNA dystrophin expression between ctr and Myo45-55, Myo45-52 at three days of differentiation (B) mRNA dystrophin expression is illustrated in OEnc44s2 experiment in the same cell lines at three days of proliferation and differentiation. We report significant difference between ctr and OEnc44s2 condition (AAV9nc44s2) but surprisingly the expression in control condition in Myo45-52 is the highest compared to ctr and Myo45-55 in opposite to expression profile illustrated above. However, similar trend is observed at 3 days of differentiation in Myo45-52 condition and Ctr myoblasts.

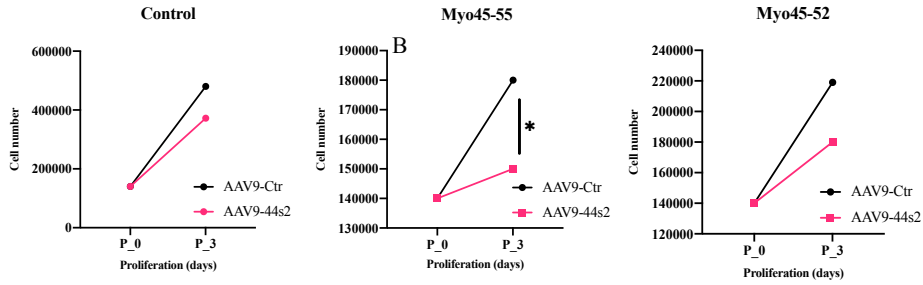
Abbreviations: ctr=control, Myo45-55=myoblasts issued from BMD Δ 45-55 patients and Myo45-52=myoblasts issued from a DMDdel45-52 patient.

lncRNA44s2 accelerates the differentiation in human primary myoblasts

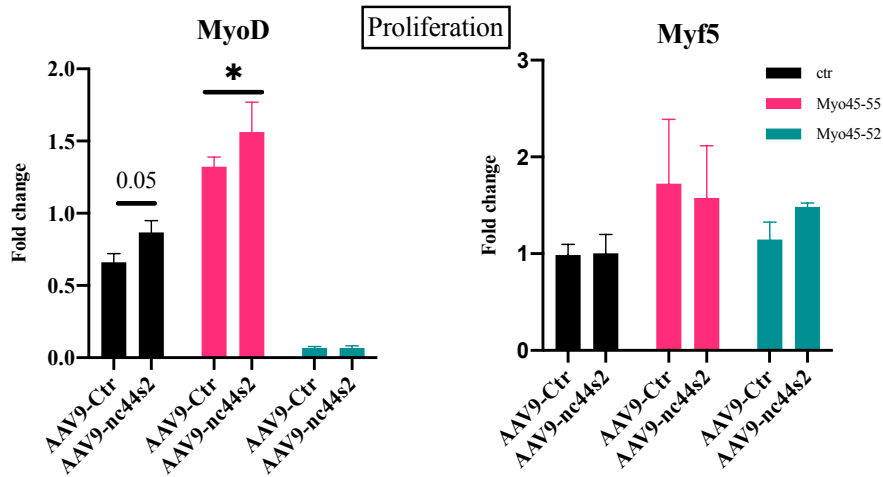
The phenotype correlation in BMD Δ 45-55 patients with the lncRNA genomic presence profile underlined the lncRNA 44s and 44s2 as two candidates of phenotype variability actors. In primary myoblasts issued from control, BMD Δ 45-55 and DMDdel45-55 patients an increase of lncRNA44s and 44s2 expression at 3 days of differentiation was observed. Based on reported results, the lncRNA44s2 was selected to assess its role during myogenesis. To do so, the lnc44s2 sequence was over-expressed (OE) in WT, Myo45-55 and Myo45-52 myoblasts using an AAV9 vector (AAV9-nc44s2) (Figure 6A). The transduction efficiency at three days of proliferation was estimated at 60% in Myo45-55 and 45% in Myo45-52 and WT cell lines (cell count of GFP+ myoblasts/ total number of myoblasts in 3 images) (Supplementary Data Figure 2A). The lnc44s2 OE was

analyzed by RT-qPCR and data confirmed a significantly higher expression of lnc44s2 in all three cell types (Supplementary data Figure2B). The lncRNA44s2 OE induced a decrease of proliferation rate in all three cell lines between treated AAV9-nc44s2 condition compared to AAV9-control (Figure 6B). In order to determine the regulatory role of lncRNA 44s2 during proliferation myomarkers expression was investigated at 3 days of proliferation. lncRNA expression profile did not change during proliferation thus, as expected, the Myf5 expression was not significantly different between control and OEnc44s2 condition (Figure 6C). However, the expression of MyoD was significantly increased in the treated AAV9-nc44s2 condition compared to AAV9-control in the Myo45-55 cell line. Despite a similar trend with the increase in the MyoD expression fold change from 0.6 compared to 0.8 in AAV9-control in the WT we did not reach statistical significance (Figure 6C).

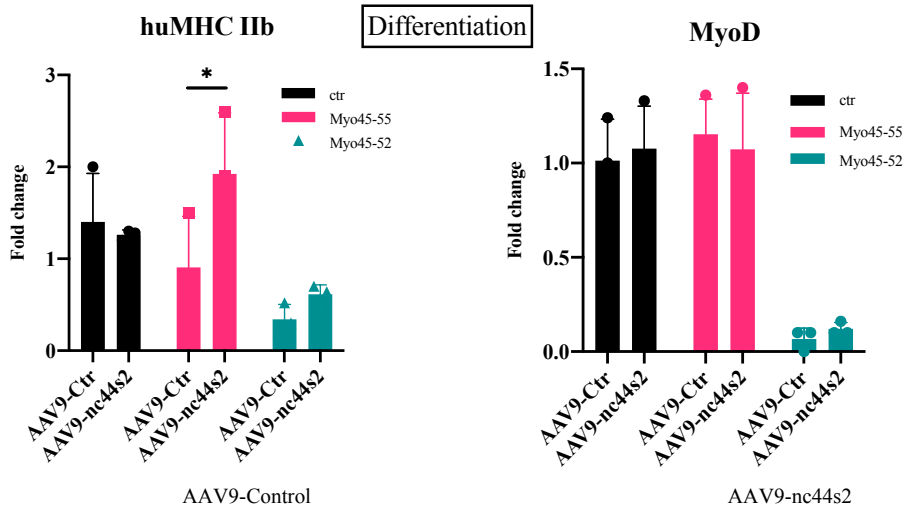
Primary human myoblasts



C



D



E

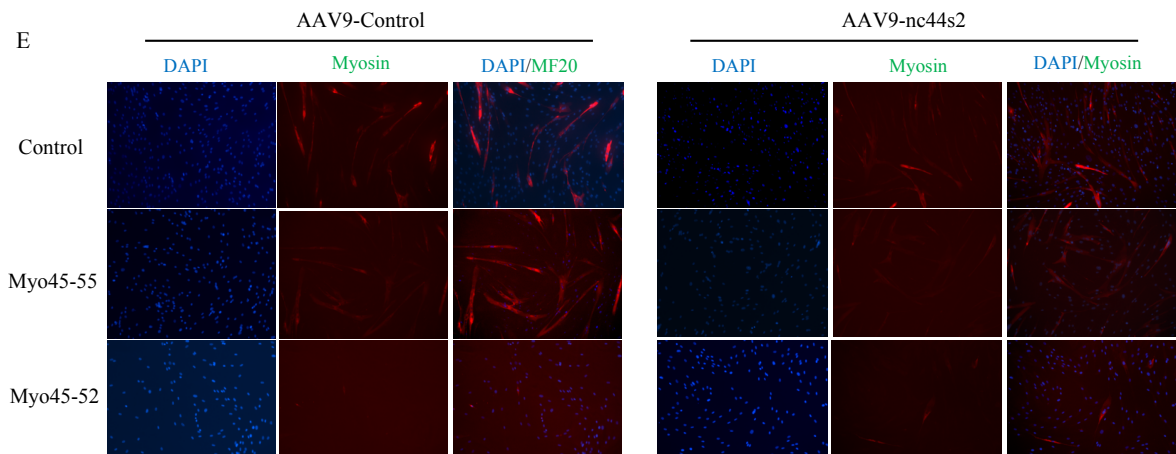


Figure 6 nc44s2 OE study during myogenesis in human primary myoblasts. (A) Experimental protocol, 3 cell lines were transduced via AAV9 vector with nc44s2 during proliferation (plating day 0, transduction at day 1(P1) and platelet harvest at day 3 (P3), for the differentiation experiment, cells were plated at day 0 and transduced at 80% of confluence, platelet harvest at day 3 (D3) (B) Growth charts depicting the proliferation rate in three cell lines, red color illustrates the AAV9 control condition, green the AAV9nc44s2 (statistically significant decrease in Myo45-55 myotubes) (C) MyoD and Myf5 expression assessment at proliferation day three (significant increase in MyoD expression in Myo45-55 $p=0.02$ by two-way ANOVA) (D) MyHCIIb and MyoD expression at 3 days of differentiation (significant increase in Myo45-55 of MyHCIIb, $p=0.03$ by two-way ANOVA) (E) Immunofluorescence experiment illustrating the myotubes at 3 days of differentiation in three cell lines, blue=DAPI, green = MF20, DAPI/MF20 visualizes at X10 magnification followed by fusion index evaluation (statistical increase in myotubes in Myo45-55/52).

In order to determine the regulatory role of lncRNA 44s2 during proliferation myomarkers expression was investigated at 3 days of proliferation. lncRNA profile did not change during proliferation thus, as expected, the Myf5 expression was not significantly different between control and OEnc44s2 condition (Figure 6C). However, the expression of MyoD was significantly increased in the treated AAV9-nc44s2 condition compared to AAV9-control in the Myo45-55 cell line. Despite a similar trend with the increase in the MyoD expression fold change from 0.6 compared to 0.8 in AAV9-control in the WT myoblasts we did not reach statistical significance (Figure 5B).

These findings suggested the lnc44s2 involvement during the differentiation and lead us to study its potential regulatory role at three days of differentiation. First, we confirmed an efficient transduction in all cell lines, the GFP+/total myoblasts count estimated a 50% rate of transduction in

Myo45-55 myoblasts, 30% in control and 50% in Myo45-52 (Supplementary data Figure 2B). The lnc44s2 OE was analyzed by RT-qPCR (Supplementary data Figure 2C). Surprisingly, we did not reach significant increase in the fold change of the lncRNA44s2 OE condition in WT and Myo45-52 cell lines at three days of differentiation despite a similar trend to the increase with 4- and 6-fold change in the lncRNA44s2 expression respectively (Figure 5C). Then, we analyzed the expression of a late differentiation myomarker, the Myosin Heavy Chain (MHC) by RT-qPCR and underlined a significant increase in the fold change of AAV9-nc44s2 condition compared to AAV9-control in the Myo45-55 and WT cell line at three days of differentiation (Figure 6D). In addition, the fusion index analysis at the same timepoint revealed a significant increase in myotubes, thus a statistically significant increased differentiation rate in Myo45-55 and Myo45-

52 AAV9-nc44s2 condition compared to AAV9-control (Figure 6E and D).

Altogether, based on the decrease of proliferation rate in treated conditions in all three cell lines, the increase of differentiation

DISCUSSIONS

In this translational study we address the question of the role of lncRNA in the BMD phenotype variability based on clinical phenotype characterization, *in silico* analysis of BMD Δ 45-55 patients and *in vitro* experiments. We selected 4 lncRNA boarding the in-frame exons 45 to 55 deletion mutation in the *DMD* gene of BMD Δ 45-55 patients. In a previous report we have described the favorable clinical phenotype characterization of the largest cohort of BMD Δ 45-55 patients (n=50) according to a Clinical Severity Scale (CSS). Here we present *in silico* analysis of the breakpoint of the deletion (+-20pb) in 19 patients BMD Δ 45-55 based on whole genome sequencing data (WGS).

Miyazaki et al. have studied the deletion breakpoint in three BMD Δ 45-55 patients and identified specific breakpoints to each patient but did not identify any significant homology between the distant and proximal

myomarkers expression at three days of differentiation and in addition the increase of fusion index in treated condition in Myo45-55 demonstrate the role of lncRNA44s2 during the differentiation process.

sequences²⁶. In this report, the WGS analysis of 19 patients reveal that family cases (n=3) have similar breakpoint and that there are two most frequent breakpoint sites (+-20pb). Interestingly only one patient had a very large deletion of 200 kb. This particular feature of the intron 44 and 55 are probably explained by the large size of the introns²⁷. Also, several previous works have identified a deletion hotspot in *DMD* gene involving exons 45-55^{19,20,26,28}.

We completed this initial data with the profile of the genomic presence of the 4 lncRNA in a total number of 38 patients (Table 1). In this study we identified that 19 patients have a specific breakpoint that preserves the lncRNA sequences localized in the introns 44, 11 patients preserve all lncRNA sequences, 7 patients have only lncRNA 44s and one patient have no lncRNA sequences (Figure 1). Also, we highlighted that there are two most frequent clusters of the lncRNA presence, one preserving the lncRNA from

intron 44 and the second preserving all lncRNA.

Interestingly, we identified a correlation of favorable mild CSS skeletal muscle phenotype and unique presence of lncRNA44s on genomic level (n=7). The CSS severity scale of the skeletal muscle phenotype confirmed the exclusive BMD phenotype in the specific BMD Δ 45-55 cohort and revealed the slowly progressive evolution of the muscular dystrophy. Therefore, we analyzed the clinical phenotype in 7 patients from the cluster with unique lncRNA44s and identified that with a mean age at 37 \pm 20 years. It appears that for these patients there are high chances to remain in a mild CSS type as their severity type was assessed at similar age when supposed to reach the intermediate CSS type (mean at 37 \pm 19 y.o). In the cluster with lncRNA44s and 44s2 the patient mean age is 39 \pm 19 and the only severe CSS patient who lost ambulation at 72 y.o. had a milder deficit in the upper limb and was autonomous for the wheelchair transfers. Altogether this information, suggest a trend to less severe phenotype in patients who preserve the lncRNA localized in intron 44. Also, the expression levels of lncRNA 44s and 44s2 were at similar levels between control and BMD Δ 45-55 muscular biopsies (n=3) but in contrary, had significantly poor expression in

BMDdel3-7(n=2- del3-5 (n=1) and DMD patients (n=3) compared suggesting a positive correlation with the less severe phenotype. The age of the collected skeletal muscle biopsy was at 38 and 58 for the BMD Δ 45-55 patients it was not documented for the last one.

Concomitantly, in vitro study of lnc44s2 overexpression in human primary myoblasts of control, Myo45-55 (BMD45-55 patient) and Myo45-52 (DMD 45-52 patient) revealed its role in the differentiation acceleration. Indeed, literature data point out the role of lncRNA in the regulation of the cellular proliferation and differentiation processes^{12,13}. It is well known that quiescent satellite cells express *Pax7* and that myogenesis is initiated by the mitosis activation and followed by the activation of the myogenic transcription factors like *MyoD*, *Myogenin*, *Myf5* and *Desmin*^{6,8,29,30}.

Recent literature data report Linc-MD1 as specifically activated during differentiation in myoblasts and satellite cells³¹⁻³³. Interestingly, Linc-MD1 is expressed in newly regenerating fiber and is abundant in dystrophic condition similar to our results of the lncRNA 44s, 44s2 and 55s,55as expression profiling in human primary and immortalized myoblasts Myo45-55 (BMD Δ 45-55 patient). Upstream of the

MyoD locus is localized the MUNC lncRNA (MyoD upstream noncoding) which initiate transcription in the DRR (distal regulatory region) RNA locus^{34,35}. Overexpression and downregulation experiments of MUNC in myoblasts result in the increase/decrease of *Myogenin*, *MyH3*. In a similar way, based on our findings lncRNA 44s2 increase the *MyoD* expression at three days of proliferation suggesting a differentiation determination. Following, the myosin heavy chain (MyHC) increase at three days of differentiation confirming the late differentiation process.

Bovolenta et al. reported the downregulation of a luciferase reporter construct carrying the minimal promoter region of the muscle dystrophin isoform by the overexpression of lncRNA from intron 44 and 55 in human rhabdomyosarcoma and neuronal cells³⁶. Interestingly, previous report detected a significant difference in the dystrophin protein quantification which varied from 50 to 80% in BMD Δ 45-55 patients³⁷. Anthony et al. underlined in their study based on various DMD mutations, including del45-55 patients (n=4) not only a variability in the BMD population but generally a positive correlation of dystrophin protein expression with clinical severity. The BMDel45-55 patients from this English cohort had a range of 60 to 90% of dystrophin protein WB

quantification. In addition, the clinical classification was mild for all four of them despite the presence in two patients of skeletal muscle deficit (patient 10 and 11)³⁸. From the previous study on phenotypic and genotypic characterization we reported a negative correlation of WB relative dystrophin quantification with the age at the diagnosis in BMD Δ 45-55 with positive skeletal muscle deficit suggesting that there are additional factors than dystrophin that are involved in triggering the functional deficit.

The therapeutic dogma is to transform severe phenotype, DMD patients into milder BMD patients^{39,40}. Actual AONs approved therapies are limited to one exon skipping. To improve that, ongoing preclinical effort is to perform multi-exon skipping^{27,28,41,42}. The complete multi-exon skipping with a combination treatment of up to 10 oligonucleotides of the large deleted exon 45-55 BMD patients revealed significant technical and practical hurdles to develop this type of therapy^{18,20,39,43}. In contrary to the selective limited number DMD patients and continuously life-time administrate OAN therapy, genome editing has the ability to make permanent changes to the *DMD* gene sequence^{23,44,45}. Ousterout et al. demonstrated the efficacy and feasibility of a gene editing strategy to restore the dystrophin reading

frame by targeting the mutational hotspot at exons 45-55 in skeletal muscle myoblasts from DMD patients²¹. In a different study, Young et al successfully corrected three DMD hiPSC lines that required ~530kb, 670kb, 725kb NHEJ of the *DMD* gene. In addition, the localization of sgRNA did not preserve any of lncRNA sequences localized in intron 44 and 55 and were localized approximately 500bp close to the exon 44 and exon 56 respectively in order to assure a small but functionally generated new intron and perform an NHEJ of up to 725kb^{46,47}. Interestingly, compared to our results, we have identified only one patient with a similar very large spontaneous *DMD* gene deletion. As the therapeutic relevance of the MES45-55 could be deduced from the clinical status of BMD Δ 45-55 patients carrying spontaneous deletion, we suggest to follow the reported breakpoints during the design of chimeric introns by MES.

These results of in vitro study in human primary myoblasts from BMD Δ 45-55 patients suggests that the lncRNA sequences localized in intron 44 and 55 could be interpreted as an indicator of the regeneration. We hypothesize that the rescue myogenesis regulated by lncRNA in dystrophic muscles will at first compensate the dystrophy. The lncRNA expression in the BMD Δ 45-55 patients is similar to a control subject, representing an early stage of the muscular dystrophy disease. With the progression of the disease, the dystrophic process will stress out the proliferation and differentiation equilibrium and the lncRNA will no longer be expressed, as seen in DMD patients muscular biopsies. Based on this data, the expression of lncRNA 44s2 can then be associated with a favorable outcome reflecting the regeneration process during which it is expressed.

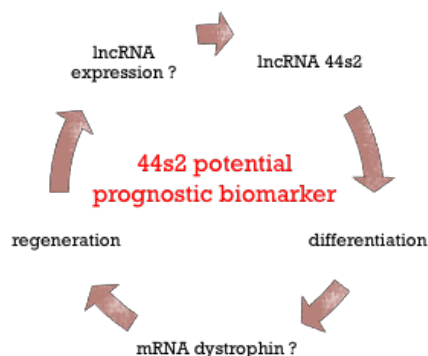


Figure 7 lncRNA role during regeneration in dystrophic skeletal muscle

On the other hand, the downregulatory role of these lncRNA on the dystrophin mRNA pointed out by Bovolenta et al. may be triggered at early stage of the disease by the dystrophin protein presence; the progressive loss of this protein would then lead to the downregulation the lncRNA expression. The advancement of the dystrophy with the

exhaustion of the pool of skeletal muscle stem cells could potentially explain the lack of expression of lncRNA 44s and 44s2 in DMD skeletal muscle biopsies. Overall, these findings suggest that lncRNA 44s2 is involved in muscle differentiation process and could be a potential disease progression biomarker.

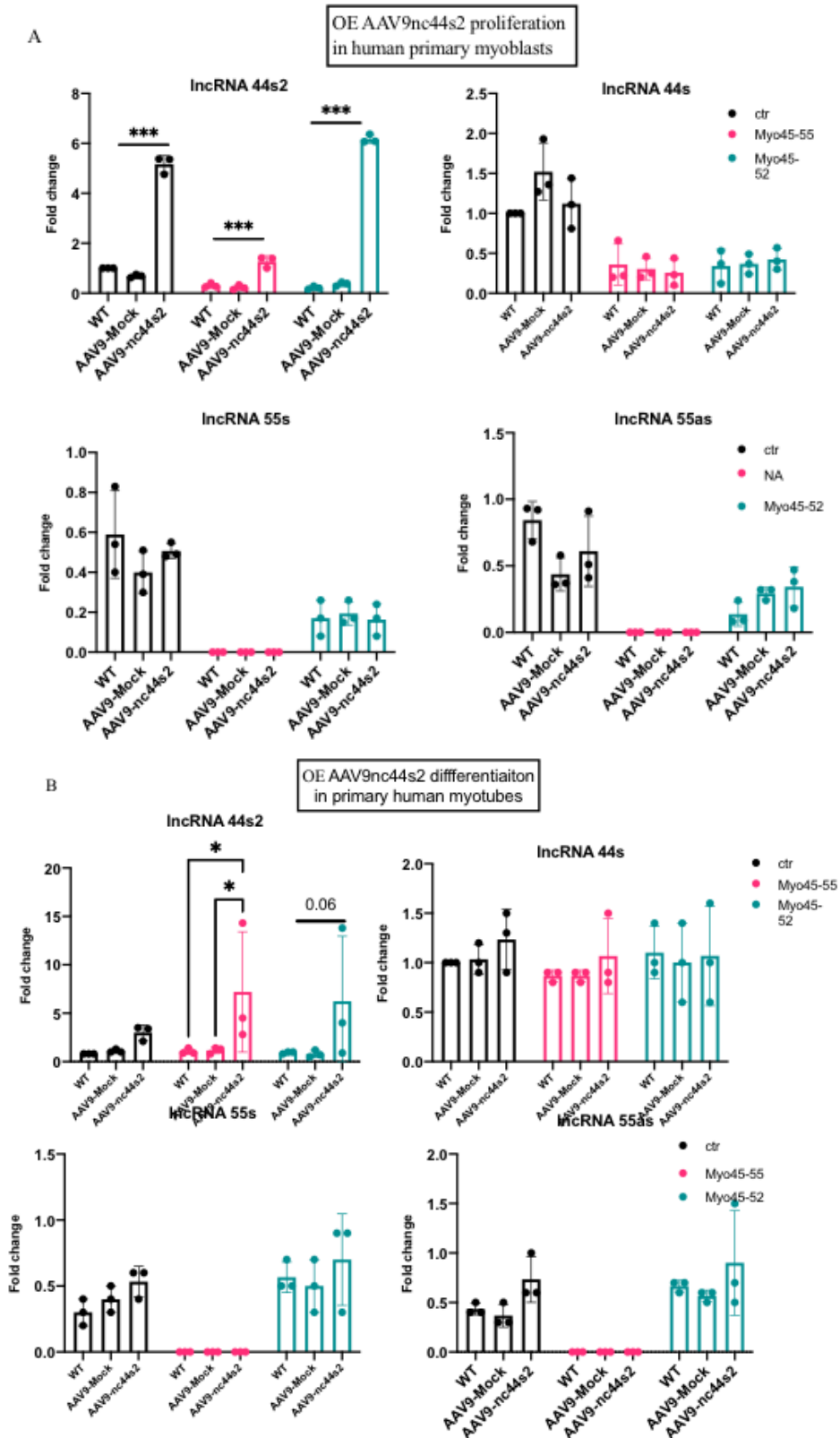
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Supplementary data



C

WGS

BREAKPOINT – DNA PCR

patient WGS	44s	44s2	55s	55AS	patient WGS	44s	44s2	55s	55 s2	55AS	patient WGS
BOOHOJT	P	P	N	PN	BOOHOJT	P	P	N	N	N	BOOHOJT
BOOHOJU	P	P	P	P	BOOHOJU	P	P	P	P	P	BOOHOJU
BOOHOJV	P	P	P	P	BOOHOJV	P	P	P	P	P	BOOHOJV
BOOHOJW	P	P	P	P	BOOHOJW	P	P	P	P	P	BOOHOJW
BOOHOJY	P	P	N	PN	BOOHOJY	P	P	N	N	N	BOOHOJY
BOOHOJZ	P	P	N	N	BOOHOJZ	P	P	N	N	N	BOOHOJZ
BOOHOK0	P	P	P	P	BOOHOK0	P	P	P	P	P	BOOHOK0
BOOHOK1	P	P	P	P	BOOHOK1	P	P	P	P	N	BOOHOK1
BOOHOK2	P	P	N	PN	BOOHOK2	P	P	N	N	N	BOOHOK2
BOOHOK3	P	P	N	PN	BOOHOK3	P	P	N	N	N	BOOHOK3
BOOHOK4	P	P	N	PN	BOOHOK4	P	P	N	N	N	BOOHOK4
BOOHOK5	P	N	N	N	BOOHOK5	P	N	N	N	N	BOOHOK5
BOOHOK6	P	P	N	N	BOOHOK6	P	P	N	N	N	BOOHOK6
BOOHOK7	P	P	P	P	BOOHOK7	P	P	P	P	P	BOOHOK7
BOOHOK8	P	P	P	P	BOOHOK8	P	P	P	P	P	BOOHOK8
BOOHOK9	P	P	N	N	BOOHOK9	P	P	N	N	N	BOOHOK9
BOOHOKA	N	N	N	PN	BOOHOKA	N	N	N	N	N	BOOHOKA
BOOHOKB	P	N	N	PN	BOOHOKB	P	N	N	N	N	BOOHOKB

D

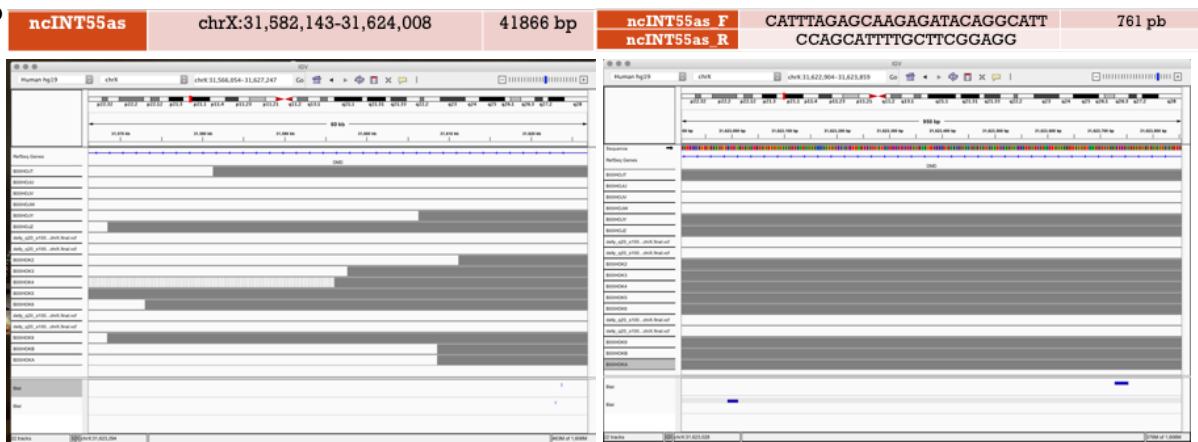


Figure 8 Supplementary data (A) Proliferation (B) Differentiation OEAAV9nc44s2 experiment (C) WGS in 19 patients and PCR validation of lncRNA intron 44 and 55 (D) Blast of lncRNA 55as primers

ARTICLE 3

Context:

During my first year of PhD I have participated to the study of miR-708 et miR-34 in the nNOS regulation in dystrophic process and I report the published article entitled:” miR-708-5p and miR-34c-5p are involved in nNOS regulation in dystrophic context”.

Abstract:

Our group previously revealed a decrease of nNOS expression in BMD patients (BMDdel45-55) all presenting a deletion of exons 45 to 55 in *DMD* gene, impacting the nNOS binding site of Dystrophin. Moreover, the degree of severity in the phenotype of these patients was correlated with the delocalization of nNOS into cytoplasm of fibers. Here, we screened the expression of 617 miRNAs in muscular biopsies of control subjects and BMDdel45-55 patients using TLDA. Four miRNAs were selected for their overexpression in BMDdel45-55 patients compared to controls and because they potentially could target the nNOS-3'UTR.

Project contribution:

I have transfected with antimiR-708 and antimiR-34 DMDdel45-55 and control myoblasts and studied resulting nNOS expression by RT-qPCR. In consequence, nNOS expression was higher in transfected conditions compared to control.

For this study, I have performed RNA extraction and RT, RT-qPCR experiments. Also, I have participated to the results interpretation and article revision.


These experiments allowed to reveal nNOS expression regulation by miR-708 and miR-34 in human immortalized myoblasts.

RESEARCH

Open Access

miR-708-5p and miR-34c-5p are involved in nNOS regulation in dystrophic context



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Abstract

Background: Duchenne (DMD) and Becker (BMD) muscular dystrophies are caused by mutations in the *DMD* gene coding for dystrophin, a protein being part of a large sarcolemmal protein scaffold that includes the neuronal nitric oxide synthase (nNOS). The nNOS was shown to play critical roles in a variety of muscle functions and alterations of its expression and location in dystrophic muscle fiber leads to an increase of the muscle fatigability. We previously revealed a decrease of nNOS expression in BMD patients all presenting a deletion of exons 45 to 55 in the *DMD* gene (BMDd45-55), impacting the nNOS binding site of dystrophin. Since several studies showed deregulation of microRNAs (miRNAs) in dystrophinopathies, we focused on miRNAs that could target nNOS in dystrophic context.

Methods: By a screening of 617 miRNAs in BMDd45-55 muscular biopsies using TLDA and an *in silico* study to determine which one could target nNOS, we selected four miRNAs. In order to select those that targeted a sequence of 3'UTR of *NOS1*, we performed luciferase gene reporter assay in HEK393T cells. Finally, expression of candidate miRNAs was modulated in control and DMD human myoblasts (DMDd45-52) to study their ability to target nNOS.

Results: TLDA assay and the *in silico* study allowed us to select four miRNAs overexpressed in muscle biopsies of BMDd45-55 compared to controls. Among them, only the overexpression of miR-31, miR-708, and miR-34c led to a decrease of luciferase activity in an *NOS1*-3'UTR-luciferase assay, confirming their interaction with the *NOS1*-3' UTR. The effect of these three miRNAs was investigated on control and DMDd45-52 myoblasts. First, we showed a decrease of nNOS expression when miR-708 or miR-34c were overexpressed in control myoblasts. We then confirmed that DMDd45-52 cells displayed an endogenous increased of miR-31, miR-708, and miR-34c and a decreased of nNOS expression, the same characteristics observed in BMDd45-55 biopsies. In DMDd45-52 cells, we demonstrated that the inhibition of miR-708 and miR-34c increased nNOS expression, confirming that both miRNAs can modulate nNOS expression in human myoblasts.

Conclusion: These results strongly suggest that miR-708 and miR-34c, overexpressed in dystrophic context, are new actors involved in the regulation of nNOS expression in dystrophic muscle.

Keywords: Duchenne muscular dystrophy (DMD), Becker muscular dystrophy (BMD), miRNA, nNOS

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Background

Duchenne muscular dystrophy (DMD) is a progressive and fatal X-linked neuromuscular disorder caused by mutations in dystrophin gene (*DMD*) [1, 2]. The disease is due to mutations that disrupt the translational reading frame, leading to the loss of the protein dystrophin expression. Mutations in the *DMD* gene that preserve the open reading frame allow the production of an abnormal truncated dystrophin still retaining some functional capacity, leading to a milder muscle disease (Becker muscular dystrophy or BMD) [3, 4]. This feature is the rationale of exon skipping therapy and genome editing strategies now in development [5–7]. The principle of these approaches is to delete one or multiple exons in order to obtain the production of a truncated dystrophin, inducing a phenotypic conversion of DMD into BMD. To apply these strategies to a larger number of eligible patients, the skipping of exons 45 to 55 of the *DMD* gene has been proposed since that could correct the reading frame in about 63% of DMD patients with deletions [8, 9]. Given the perspective of this approach, the therapeutic relevance of the resulting 45–55 truncated dystrophin may be deduced from the clinical status of BMD patients carrying spontaneous deletion of exons 45 to 55 of the *DMD* gene (BMDd45-55). Likewise, molecular investigations of factors involved in pathophysiological process occurring in muscle of these patients are of great interest.

Dystrophin is a 427-kDa protein that links the cytoskeleton to sarcolemma via the dystrophin-associated protein complex (DAPC) [10]. DAPC provides stability and integrity to the muscle membrane during contraction. The loss of dystrophin leads to a breakdown of the DAPC complex, and as consequences, the muscle fibers become more sensitive to mechanical stresses, leading to muscle degeneration, chronic inflammation, or increased fibrosis [11, 12]. Among the partners of the dystrophin, the neuronal nitric oxide synthase (nNOS), that synthesizes nitric oxide (NO), was shown to play critical roles in a variety of muscle functions, including not only contraction, regeneration, atrophy, glucose uptake, and blood perfusion [13] but also transcriptional regulation [14]. Indeed, NOS enzymatic activity was recently demonstrated as essential for the rescue of muscle mass after atrophy induced by unloading [15], as well as in reducing the extent of atrophy during disease [16], and these effects were mostly assigned to activation of muscle stem cells by the NO production. Three different isoforms of nNOS, namely nNOS α , nNOS β , and nNOS μ , were described to be expressed in the skeletal muscle. The nNOS μ , the major one, contains a PDZ domain which allows its binding to the rod domain of the dystrophin at the spectrin-like repeats 16 and 17 (R16/17) encoded by exons 42–45 [17]. It has been shown that in the absence of dystrophin, nNOS μ was delocalized from the sarcolemma of the muscular fibers and its expression decreased [18, 19]. Alteration of nNOS μ expression and

location was described to contribute to DMD pathophysiology by the disturbance of NO signaling leading to an increase of the muscle fatigability [20, 21].

Our goal was to identify the molecular factors that can modulate the expression of nNOS and the muscular biopsies of BMDd45-55 patients that were sought to be a pertinent tool. Indeed, in these patients, the deletion of the 45–55 exons in dystrophin mRNA should partially delete the spectrin motif repeat 17 in the resulting protein and could alter the nNOS μ anchoring. A previous study revealed that the BMDd45-55 patients displayed variable clinical and histological phenotypes and that a subsequent decrease of nNOS protein expression occurred in these patients compared to healthy subjects [22]. Furthermore, several studies demonstrated a deregulation of miRNA expression profiles in dystrophinopathies [14, 23–26]. Cacchiarelli et al.'s study showed also that the loss of nNOS sarcolemmal localization leads to the deregulation of the expression of several microRNAs (miRNAs) [14]. miRNAs are short noncoding RNA that regulate mRNA post-transcriptionally either by promoting mRNA degradation or by inhibiting protein translation [27]. miRNAs have been shown to regulate functions of the skeletal muscle both in normal and pathological states [14, 28–30]. Altogether, these studies suggest a link between miRNA expression, nNOS expression, and physiopathology of dystrophinopathies. Thus, the aim of the present study was to identify miRNAs that could modulate nNOS expression by screening the miRNA profile in BMDd45-55 muscular biopsies.

Methods

Ethics approvals

Muscle biopsies were collected from patients after informed consent, and this study was performed with agreement from the Committee for the Protection of Persons (CPP) concerned.

Cohort of patients

Nine Becker muscular dystrophy (BMD) patients characterized for a deletion of exons 45–55 of the *DMD* gene were studied. These patients were already described [22]. Indeed, the clinical status of the patients was scored using the Gardner–Medwin and Walton scale (GMWS) [31], and the histopathological status based on routine hematoxylin and eosin (HE) staining of muscle cryosections has been investigated showing a large histological disparity. These criteria allowed defining three classes of severity: (i) “mild” (GMWS ≤ 2 [i.e., still able to normally climb stairs] and normal muscle biopsy); (ii) “moderate” (GMWS ≤ 2 or mild dystrophic muscle biopsy); (iii) “severe” + (GMWS ≤ 2 and/or dystrophic muscle biopsy) (Table 1). The 9 patients were biopsied at an age ranging from 9 to 69 years old for diagnostic purposes after

Table 1 Clinical and histopathological phenotypes compared with nNOS μ expression in BMDd45-55 patients

Name	Severity class	Age at muscle biopsy	Histopathological status (*)	nNOS μ protein expression (**)
Ctrl 1		26	N	++
Ctrl 2		40	N	++
Ctrl 3		10	N	++
Ctrl 4			N	++
Ctrl 5			N	++
Patients				
BMD 1	Moderate	35	+/- (35)	+/-
BMD 2	Severe +	13	+ (13)	+
BMD 4	Severe +	33	++ (33)	+
BMD 7	Moderate	40	+/- (40)	+
BMD 8	Moderate	12	+/- (12)	+/-
BMD 11	Mild	69	N (69)	+
BMD 12	Moderate	18	+/- (18)	+/-
BMD 18	ND			
BMD 31	ND			

(*) N normal, +/- mild dystrophy, + and ++ severe dystrophy, ND not determined, (**) +/- traces or not detectable in Western blot, + weak, ++ normal

informed consent, and these biopsies were used during the experiments in the present article. In addition, the five muscle biopsies used as healthy control muscles (Ctrl) were recovered as surgical wastes from orthopedic surgery of individuals without neuromuscular diseases. All human muscle biopsies were flash frozen in isopentane cooled in liquid nitrogen and evaluated for dystrophin and nNOS expression by Western blotting [22].

Taqman Low-Density Array (TLDA)

Total RNA (including miRNA and mRNA) were extracted from about 30 mg of muscular biopsy using the NucleoSpin \circledR miRNA kit from Macherey-Nagel. Total RNA (200 ng) was reverse-transcribed with the Megaplex Primer Pools A and B (human version 3), and miRNAs were quantified after a pre-amplification step, with TaqMan Array MicroRNA Cards A and B (human version 3) on the 7900HT Real-Time PCR System (AB) according to the manufacturer's guidelines. Relative quantification was performed with 2^{-dCT} method, using the mean of all miRNA expressed as normalizer.

miR-target predictions

The ability of candidate miRNAs to target *NOS1-3'*UTR was evaluated with Diana-microT algorithm and TargetScan v6.2.

Individual RT-qPCR

Thirty milligrams of muscular biopsies from 3 healthy subjects and patients BMD1, 2, 4, 8, and 11 was extracted using the NucleoSpin \circledR miRNA kit (Macherey-Nagel). One hundred nanograms of RNA was reverse-transcribed with Universal cDNA synthesis kit II (Exiqon) according

to the manufacturer's instructions. Complementary DNAs (cDNAs) were analyzed by real-time quantitative PCR performed on Light Cycler 480 instrument (Roche) using Exilent SybR Green Master Mix (Exiqon). LNA $^{\text{TM}}$ PCR Primers set from Exiqon were used for miRNA expression analysis (miR-212: 204170, miR-708: 204490, miR-34c: 205659, miR-31: 204236). miRNA expression was normalized on miR-30b-5p expression (204765) using 2^{-dCT} method.

Luciferase assay

Genomic DNA was extracted from biopsies of healthy subject using NucleoSpin \circledR Tissue kit from Macherey-Nagel, following the manufacturer's instructions. gDNA was eluted after incubation of the silica membrane 3 min, twice in 50 μ L of Elution Buffer BE, followed by centrifugation 1 min at 11,000g, being 100 μ l of total gDNA.

*NOS1-3'*UTR (Ensembl: ENST00000618760) was cloned downstream of Firefly luciferase gene in HSVTK-Luc3' modified plasmid. As this 3'UTR is 7183 pb in length, it is too large to be fully cloned in this plasmid. Therefore, we cut it in 4 overlapping parts using following primers that add restriction sites (in bold) on gDNA fragments (Table 2).

Fragments were amplified using Mastermix Phusion with the following protocol: 98 $^{\circ}$ C 30s; 10 cycles of 98 $^{\circ}$ C 10s, 58 $^{\circ}$ C 30s, and 72 $^{\circ}$ C 1 min; and 20 cycles of 98 $^{\circ}$ C 10s, 61 $^{\circ}$ C 30s, 72 $^{\circ}$ C 1 min, and 72 $^{\circ}$ C 10 min.

Amplicons were purified with NucleoSpin \circledR Gel and PCR cleanup from Macherey-Nagel. 3'UTR parts were then cloned in HSVTK-Luc3' modified plasmid between XbaI and EcorV sites.

Table 2 Primers used to fragment NOS1-3'UTR

Position in 3'UTR	Forward primer	Reverse primer
Part 1 (1-1896)	F1-5' TTGCTAGACTGGACCTCTGCCAGC -3'	R1-5' AAGGATATCCAGGGGAAATGGGATTAAGG -3'
Part 2 (1773-3685)	F2-5'- AACCTAGACTATGACTCACCTTGCTCTGC -3'	R2-5'- ATCGATATCCTTACATGCTCCCTGTCCGTG -3'
Part 3 (3607-5523)	F3-5'- AATCTAGACTGGTAGCTTCTGGAAGTAAG -3'	R3-5'- AATGATATGCCACAAGGCAGGGACTGGC -3'
Part 4 (5358-7149)	F4-5'- TAGCTAGAGAAACACAGGTCTGAGGGTCTG -3'	R4-5'- CCGATATCATTGTAACATAATGCAACAAGC -3'

Added restriction sites are indicated in bold characters (XbaI and EcoRV)

Each 3'UTR construction (24.5 ng) was co-transfected in 293T-HEK cells with 25 pg of either miR-negative control (AM17111, Ambion) or miR-212, miR-31, miR-34c, or miR-708 (AM17100, Ambion) using lipofectamine 2000 diluted in Optimem reduced medium. The plasmid CMV-Renilla luciferase (0.25 ng) was also transfected in each condition as normalizer. Five hours post-transfection, Optimem reduced medium is replaced with DMEM added with FBS 10%. Twenty-four hours after transfection Firefly and Renilla luciferase luminescences were quantified with Dual-Glo Luciferase Assay System (according to the manufacturer's instructions) on Flexstation 3 Microplate reader. Firefly luciferase activity was normalized on Renilla luciferase activity.

Human myoblast transfection

Human immortalized myoblasts from a healthy subject (ctrl) and from a DMDd45-52 patient (DMDd45-52) were used [32]. Myoblasts were plated 48 h before transfection at 3×10^4 /well of 6-well plate or 6×10^3 /well of 24-well plate in proliferation medium composed of DMEM supplemented with 5 µg/ml of insulin, 5 ng/ml of EGF, 0.5 ng/ml of bFGF, 0.2 µg/ml of dexamethasone, 25 µg/ml of fetuin, 20% of fetal bovine serum, and 16% of medium 199. Cells were transfected with 12.5 pg of either miR-negative control (AM17111, Ambion); miR-31, miR-34c, or miR-708 (AM17100, Ambion); or anti-miR-34c or anti-miR-708 (AM17000, Ambion) using lipofectamine 2000 diluted in Optimem reduced medium. Twenty-four hours after transfection, transfection medium was replaced with proliferation medium for 24 h.

miRNA expression

Cells were harvested in 300 µl of Buffer ML (NucleoSpin® miRNA, Macherey-Nagel). Total RNA (small + large RNA) was extracted from lysed cells with NucleoSpin® miRNA kit (Macherey-Nagel) following the manufacturer's instructions. cDNA generated with Universal cDNA synthesis kit II (Exiqon) according to the manufacturer's instructions was analyzed by real-time quantitative PCR performed on Light Cycler® 480 instrument (Roche) using Exilent Sybr Green Master Mix (Exiqon). LNA™ PCR Primers set from Exiqon were used for miRNA expression analysis (miR-708: 204490, miR-34c: 205659,

miR-31: 204236). miRNA expression was normalized on SNORD44 expression (203902) using $2^{-\Delta\Delta CT}$ method.

Western blotting

For nNOS detection, cells were lysed in 50 µl of RIPA buffer (150 mM NaCl, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, 5 mM ethylene diamine tetra acetic acid, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 1 mM PMSF with a mix of protease inhibitors (Roche), and centrifuged 10 min 1500g at 4 °C.

Protein extracts (20 µg) were denatured in Laemmli buffer 2× added of 10% of 2-mercaptoethanol 30 min at room temperature (RT) and incubated 15 min in ice and then 15 min at RT. Proteins were resolved by SDS-PAGE (4–12%, Invitrogen) and transferred to nitrocellulose. Membranes were blocked in tris-buffered saline 0.1% Tween-20 with 5% non-fat dry milk 1 h at RT and incubated overnight at 4 °C, with rabbit polyclonal nNOS antibody (R-20, Santa Cruz, 1:100) or with mouse monoclonal GAPDH antibody (MAB9748, Tebu-Bio, 1:8000). After being washed in TBS 0.1% Tween, membranes were incubated for 1 h at RT with secondary antibodies: goat anti-rabbit-horseradish peroxidase (HRP) (1/50000) or sheep anti-mouse HRP (1/15000) (Jackson ImmunoResearch). Western blots were revealed with enhanced chemiluminescence (Thermo Scientific) with Image Quant LAS 4000 system (GE Healthcare Life Sciences).

Immunostaining

Cells in 24-well plate were washed with PBS and fixed with paraformaldehyde 4% 10 min at RT and washed 3 times in PBS. Fixed cells were permeabilized with 0.5% Triton X-100 (Sigma-Aldrich), washed, and blocked in PBS/5% bovine serum albumin (BSA) for 40 min at RT. Cells were then incubated in PBS/1% BSA/0.1% saponin with a goat polyclonal anti-nNOS antibody (Ab1376, Abcam, 1:500), overnight at RT; washed in PBS/1% BSA/0.1% saponin; and incubated for 1 h with secondary antibody: Donkey anti-goat (Alexafluor 594 conjugate, Life Technologies, 1:500) and with DAPI (1:5000, Sigma). Fixed cells were then thoroughly washed in PBS/1% BSA/0.1% saponin and then in PBS and mounted in Fluoromount (Southern Biotech). Images were acquired

with Leica DM2500 confocal microscope using $\times 63$ objective.

Statistical analysis

Statistical analysis were performed using Student’s *t* test. A value of $p < 0.05$ was considered statistically significant.

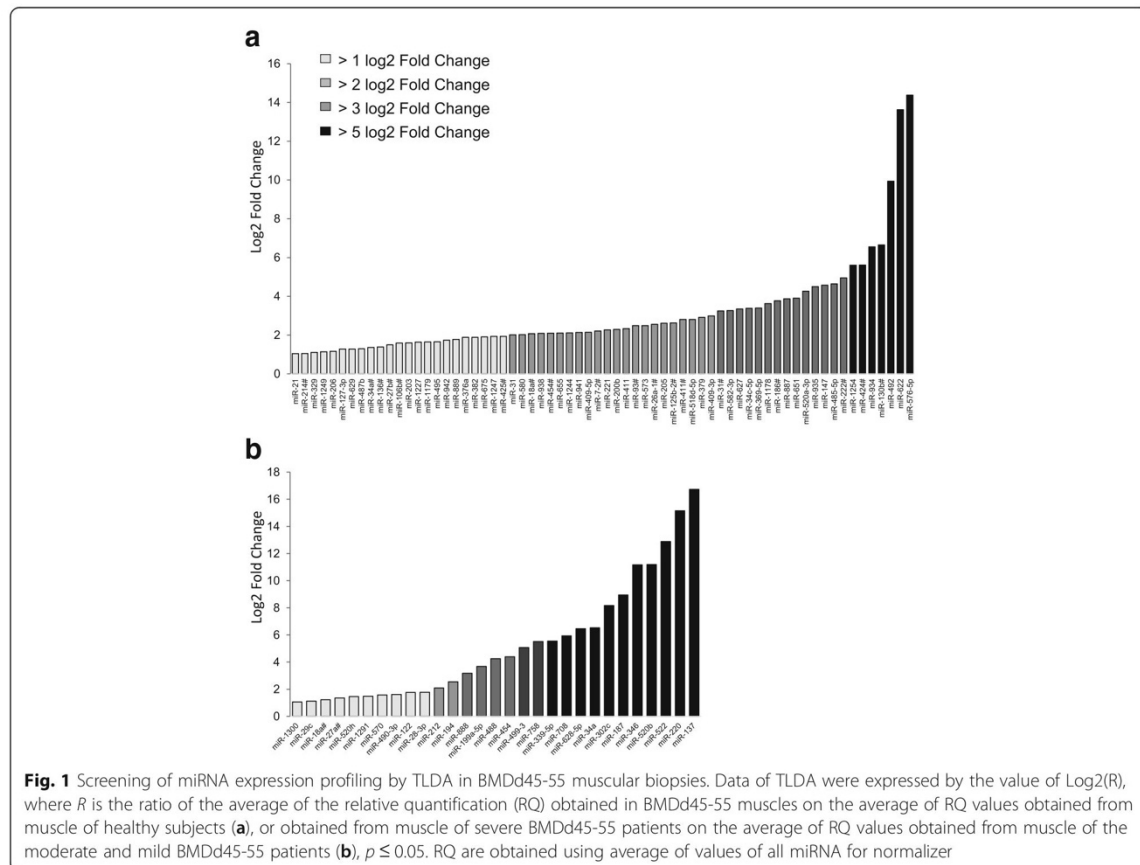
Methods used for the Additional file 4: Figure S1 are described in Additional file 1.

Results

miRNA expression profiling in BMDd45-55 muscular biopsies

To start our analysis, we took advantage of having a collection of muscle biopsies of Becker patients (BMDd45-55) bearing an in-frame deletion of exons 45 to 55 in the *DMD* gene and well characterized from a genetic point of view [22]. This collection has been the subject of a preliminary study which showed notably a decrease in the expression of the protein nNOS in the muscle of the studied patients [22]. We examine here a potential role of miRNA in the regulation of nNOS expression by investigating the expression levels of 617

miRNAs using Taqman Low-Density Array (TLDA) in muscle biopsies of 9 BMDd45-55 patients, compared to 5 control subjects (Table 1). From TLDA data (Additional file 2), we established a list of miRNAs overexpressed in the muscles of BMDd45-55 patients with the criteria of a fold change higher than 2 and a *p* value less than or equal to 0.05. By comparing miRNA expression levels between BMDd45-55 and healthy muscles as control (Fig. 1a), a total of 66 miRNAs were identified based on the defined criteria of fold change and *p* value. Furthermore, the TLDA data were also analyzed by comparing the level of miRNAs expressed in muscles of severe patients with those expressed in muscles of all the other patients (Fig. 1b). This analysis allowed the identification of 29 overexpressed miRNAs. It should be noted that none of these 29 miRNAs were found in the list of miRNAs overexpressed in BMD muscles compared to healthy subjects (Fig. 1a), probably because of the too small number of severe muscle biopsies preventing the fold change value from being statistically significant when included to the values obtained for all the BMD patients.



miR-708-5p, miR-31-5p, and miR-34c-5p target 3'UTR sequences of *NOS1* gene

To select miRNAs that could modulate nNOS expression, the total sequence of the 3'UTR of the *NOS1* gene (*NOS1*-3'UTR) was submitted to two predictive software, i.e., TargetScan Human and microRNA.org, that process alignment of the target sequence with human miRNA databases (Fig. 2a). From this study, 12 and 24 miRNAs were identified by the 2 predictive software, respectively. Surprisingly, there was no common miRNAs between the two lists. By combining the previous TLDA analysis criteria and the *in silico* investigation data, 4 miRNAs named miR-31-5p (miR-31), miR-708-5p (miR-708), miR-34c-5p (miR-34c), and miR-212-3p (miR-212) were finally selected. Overexpression of these 4 miRNAs was then validated by performing individual RT-qPCR on 5 BMDd45-55 and 3 healthy muscular biopsies (Fig. 2c). A higher level of expression of the 4 miRNAs was detected in BMDd45-55 compared to control muscles with a fold change of 6.6, 4.4, 10.1, and 3.3 for miR-31, miR-708, miR-34c, and miR-212, respectively, confirming the results obtained by TLDA (Fig. 2b, Additional file 2). Furthermore, by analyzing the sequence of the *NOS1*-3'UTR regarding the 4 selected miRNAs, we identified 5 sequences as potential targets of miR-31, 5 for miR-708, 9 for miR-34c, and 3 for miR-212 (Additional file 3: Table S1 and Fig. 3a). Their ability to bind *NOS1*-3'UTR was then tested *in vitro* using the luciferase reporter gene. If a miRNA interacted with *NOS1*-3'UTR, we would measure a decreased luciferase signal. Nevertheless, the *NOS1*-3'UTR being 7165 pb in length, it is too large to be fully cloned. Therefore, our strategy was to design 4 sequences (parts #1, #2, #3, and #4) which succeed one another with overlapping avoiding a miRNA-binding sequence being lost and covering all the *NOS1*-3'UTR sequence (Fig. 3a). Each part was sub-cloned in a plasmid downstream of the luciferase gene, and each of the 4 plasmids was co-transfected in HEK293T cells with one candidate or a non-specific control miRNA mimic. This strategy would also provide a more detailed information about the sequence of *NOS1*-3'UTR implicated in miRNA interaction. Our data showed a significant decrease of luciferase activity when the part #2 was co-transfected with the miR-31 and the part #3 with the miR-708 and when the parts #1, or #3, or #4 were co-transfected with the miR-34c. Nevertheless, no decrease of the reporter gene was observed when miR-212 was co-transfected with the parts #1, #2, #3, nor #4. These results demonstrated that miR31, miR-708, and miR34c, but not miR-212, were able to target *NOS1*-3'UTR sequences leading to a decrease of the reporter gene Firefly luciferase expression.

Altogether, these results demonstrate that these 4 miRNAs were overexpressed in the muscles of BMDd45-55

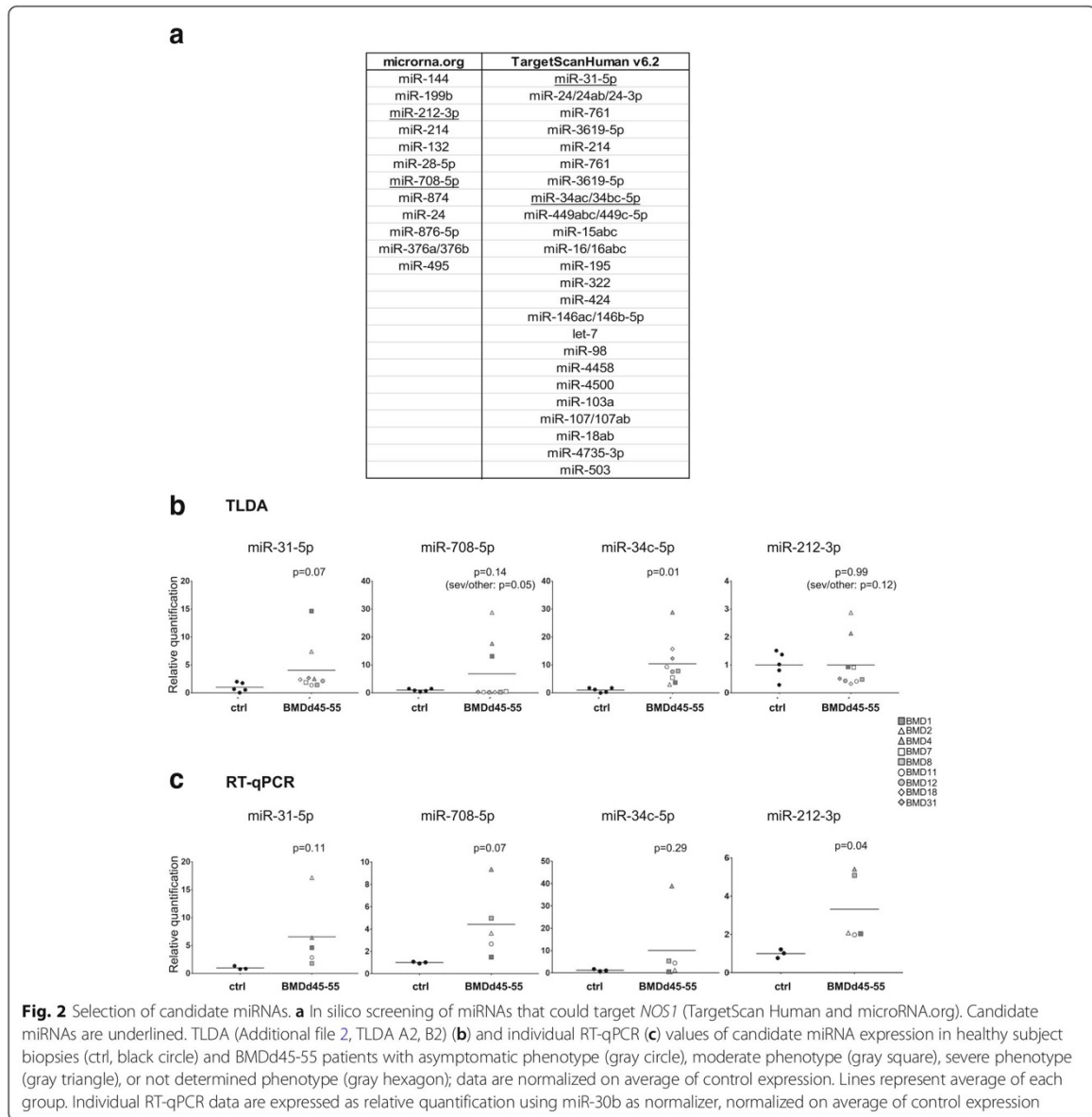
patients compared to control muscle or in severe patients compared to other patients and that only 3 of them could target sequences present in the *NOS1*-3'UTR and modulate reporter gene activity.

miR-31, miR-708, and miR-34c effect on nNOS expression in human myoblasts

In order to address the causal relationship between the overexpression of the 3 selected miRNAs and the nNOS expression in muscular context, we carried out experiments using immortalized human myoblasts from healthy subject (control) and from a patient displaying a deletion of the exons 45 to 52 (DMDd45-52) in the *DMD* gene [29]. First, these DMDd45-52 myoblasts were validated as an appropriate cellular model regarding the expression of the 3 selected miRNAs. Quantification by RT-qPCR confirmed a higher level of expression for miR-31, miR-708, and miR-34c in DMDd45-52 cells compared to control with a fold change of 2.2, 2.2, and 3.8, respectively (Fig. 4a). Furthermore, the expression of nNOS protein was investigated by Western blot and showed a significant decrease in DMDd45-52 compared to control cells (Fig. 4b). Additionally, immunostaining experiments, allowing the detection of the protein nNOS in the cytoplasm and into the nucleus of muscle cells, confirmed that nNOS staining was weaker in the DMDd45-52 compared to the control myoblasts (Fig. 4c). Overall, these results were consistent with those obtained on BMDd45-55 muscle biopsies, namely a higher level of miR-31, miR-708, and miR-34c and a decrease in the expression of nNOS, thus allowing the use of these DMDd45-52 myoblasts as a suitable *in vitro* cellular model.

To evaluate the effects of the miR-31, miR-708, or miR-34c on the nNOS expression, each of them was transfected in control myoblasts (Fig. 5a). Overexpression of the miRNAs was verified by RT-qPCR (Fig. 5a). The location and expression of nNOS protein were first investigated by immunostaining on the transfected myoblasts (Fig. 5b). Analysis of the pictures showed a decrease of the nNOS labelling in the nuclei of cells overexpressing miR-708 or miR-34c. However, no effect on nNOS expression and location could be observed when miR-31 was overexpressed compared with myoblasts transfected with the non-specific control miRNA. The reduction in the nNOS level was confirmed by Western blot experiments showing a decrease of about 30% of nNOS expression in cells overexpressing miR-708 or miR-34c, while no significant decrease could be observed in overexpressing miR-31 (Fig. 5c). Altogether, these results demonstrated that miR-708 or miR-34c could modulate nNOS expression in human healthy myoblasts.

In DMDd45-52 myoblasts, miR-708 and miR-34c expressions increased and nNOS expression decreased

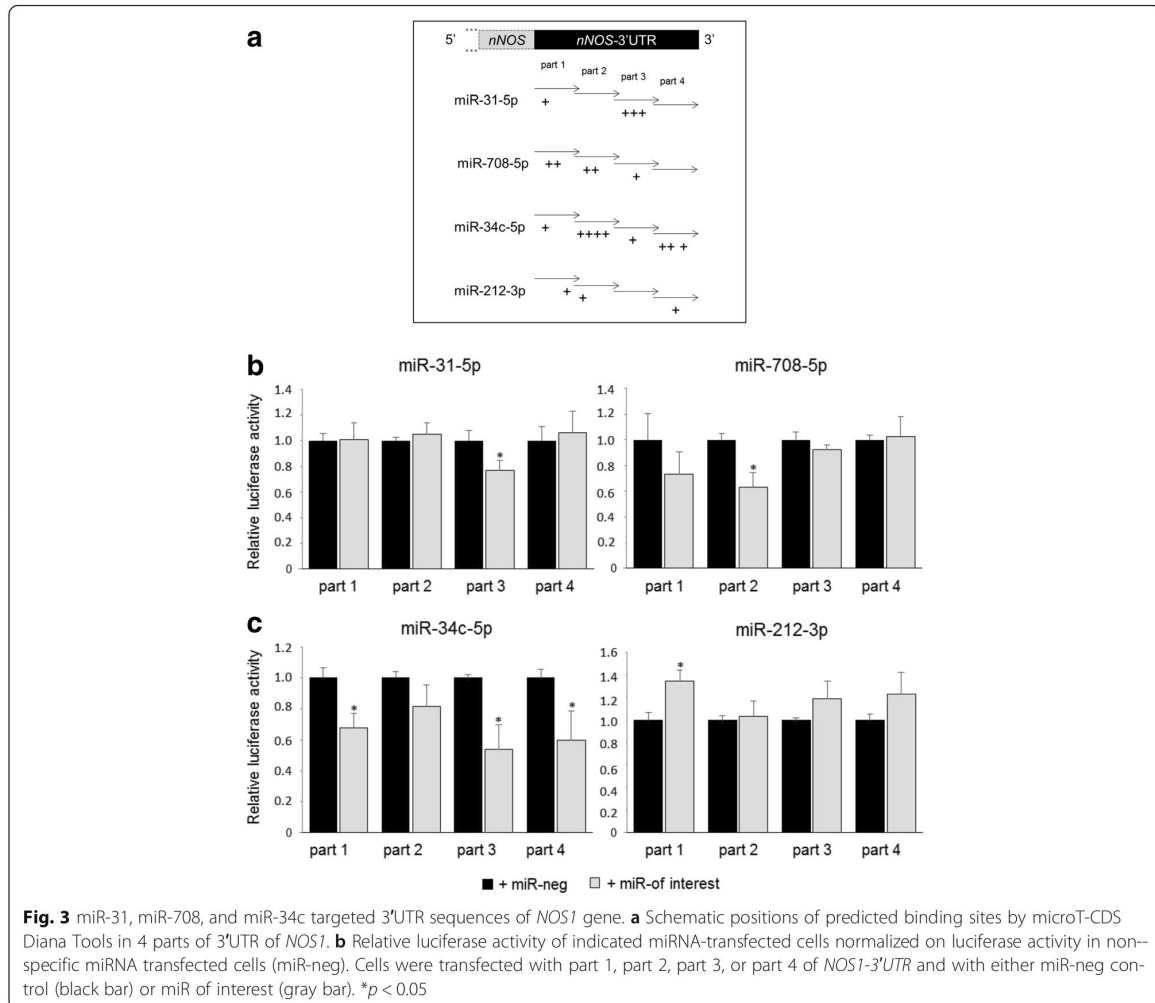


compared to control myoblasts (Fig. 4); we thus investigated in these cells the consequences of an inhibition of the miR-708 or the miR-34c by using specific antisense oligonucleotides (antimiR-708 or antimiR-34c) on the nNOS expression level (Fig. 6). The inhibition of miR-708 or the miR-34c levels by their antimiRNAs was validated by RT-qPCR experiments (Fig. 6a). In these cells, the nNOS location and expression were also investigated. Immunofluorescence experiments showed an increased staining in the nuclei of cells in which the miR-708 or the miR-34c were inhibited compared to cells

transfected with a non-specific control miRNA (Fig. 6b). These results were confirmed by Western blot experiments that showed a significant increase of 2.2 of nNOS expression in cells transfected with antimiR-708 or antimiR-34c (Fig. 6c).

Discussion

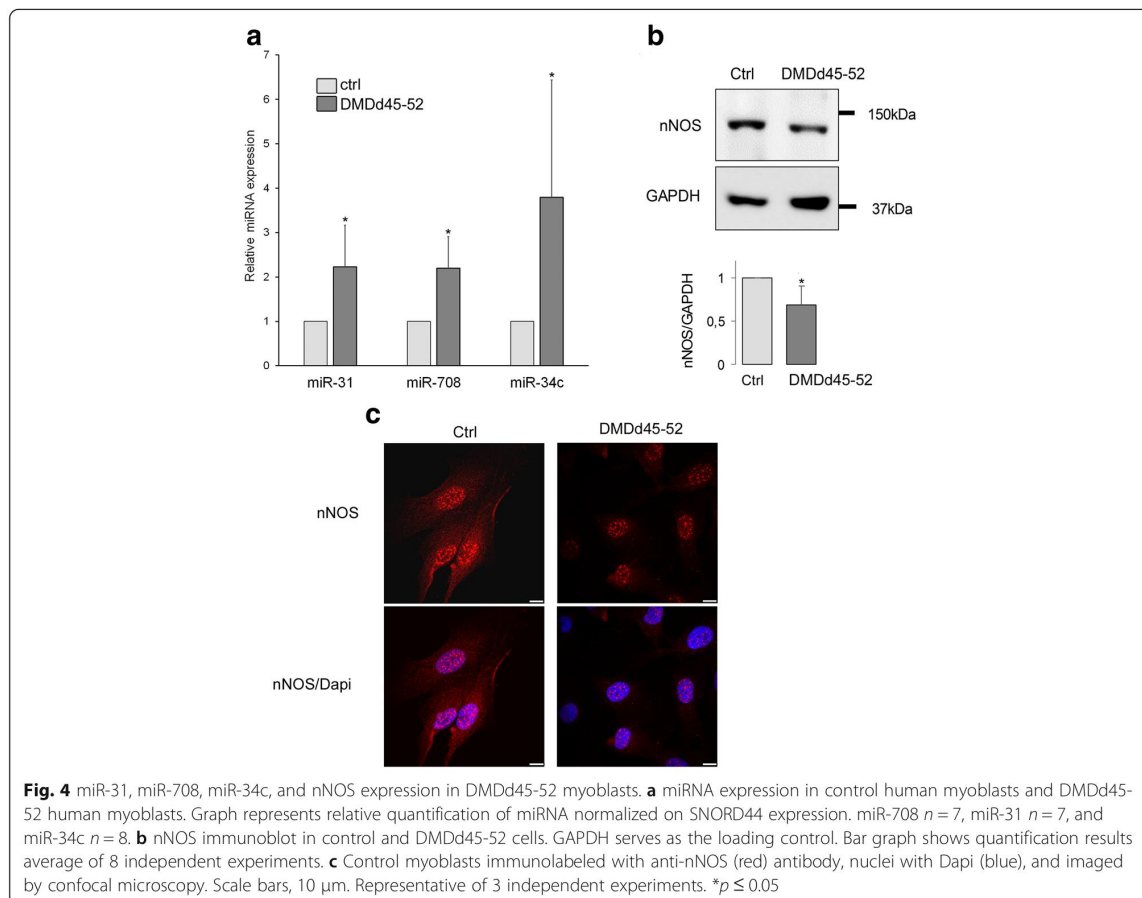
In this study, we used a variety of bioinformatic, molecular, and cell biological methods to demonstrate the role of miRNAs in driving nNOS expression. We selected 4 miRNAs (i.e., miR-31, miR-708, miR-34c, and miR-212)



since they were overexpressed in muscular biopsies of BMDd45-55 patients compared to healthy subjects or in muscular biopsies of patients with severe phenotypes compared to other patients. We then determined, *in silico*, that these miRNAs could target sequences in *NOS1*-3'UTR. A luciferase reporter study validated the targeting of *NOS1*-3'UTR by miR-31, miR-708, and miR-34c. Finally, we validated the effects of the candidate miRNAs in myoblasts. The experiments were carried out on myoblasts which were a more homogeneous cell population than those of myotubes, from which we never observed 100% of differentiated myotubes and for which efficacy of transfection experiments with miR and antagomiR was much more effective than on differentiated cells. We thus demonstrated that miR-708 and miR-34c could decrease nNOS expression in human healthy myoblasts and that their inhibition led to an increase of this protein in DMDd45-52 human cells.

Several studies showed a deregulation of miRNA expression in muscles of DMD or BMD patients [14, 23, 33] or in serum of DMD patients [26, 34]. Eisenberg et al. studied miRNA profile expression in 10 muscular diseases, and they showed an upregulation of 5 common miRNAs in these diseases [33]. They showed also a particular miRNA expression profile shared by DMD patients and severe BMD patients but not with moderate BMD patients.

Among the selected miRNAs in our study, miR-31 was already shown to be overexpressed in *mdx* mice and in muscular biopsies of DMD patients [14, 23, 35]. We found here the same results in muscular biopsies of BMDd45-55 patients, in DMDd45-52 myoblasts, and in TA muscle of *mdx* mice (data not shown). Unlike our results, Cacchiarelli and colleagues did not observe an increase of miR-31 expression in the biopsies of BMD patients. However, no information on the *DMD* gene mutations and/or phenotypes was given for the patients



included in Cacchiarelli et al.'s study. As we found a higher expression of miR-31 in severe phenotypes than in moderate phenotypes (Fig. 2c), we assume that Cacchiarelli et al.'s patients had moderate phenotypes and therefore might not exhibit a high level of miR-31. The fact that miR-31 could target nNOS by mRNA decay was described in human atrial myocytes from patients with atrial fibrillation [36]. In this study, the precise targeted sequence was identified, and it appears to be the same that we identified by the system of cloning *NOS1-3'UTR* downstream luciferase reporter gene setup in our study (Fig. 3). Surprisingly, our data revealed a slight decrease of nNOS expression by miR-31 overexpression in control human myoblasts. One reason could be the level of miR-31. Indeed, in Reilly et al.'s work, miR-31-fold increase was 2×10^4 compared to control condition whereas in our study, miR-31 increased by a factor of 4×10^3 (Fig. 5a) and therefore non-sufficient to exhibit a significant effect. However, we could not transfect more miR-31 because of deleterious effect of transfection on human myoblasts.

Nevertheless, one has to consider that the complex regulation displayed by non-coding RNAs might be different according to the studied tissues.

Concerning miR-708 and miR-34c, our results showed an effect of these two miRNAs on nNOS expression in human healthy and DMDd45-52 myoblasts. miR-708 is mostly described in cardiac muscle, where it was proposed to be involved in myocardium regeneration. Indeed, its overexpression in newborn rodents leads to the differentiation of cardiac progenitors to cardiomyocytes by targeting *MAPK14*, a cell cycle gene [37]. Otherwise, miR-708 expression is decreased in murine myoblasts atrophied by dexamethasone treatment, suggesting that miR-708 is involved in muscular development [38]. For miR-34c, several studies described it as overexpressed in *mdx* mice and in DMD patients [23, 35]. Our data were in the same way as miR-34c is overexpressed in BMDd45-55 muscle biopsies, in DMDd45-52 myoblasts, and in *mdx* mice (data not shown). This miRNA is shown to be a promoter of differentiation of murine myoblasts targeting *YY1*, a transcription factor involved

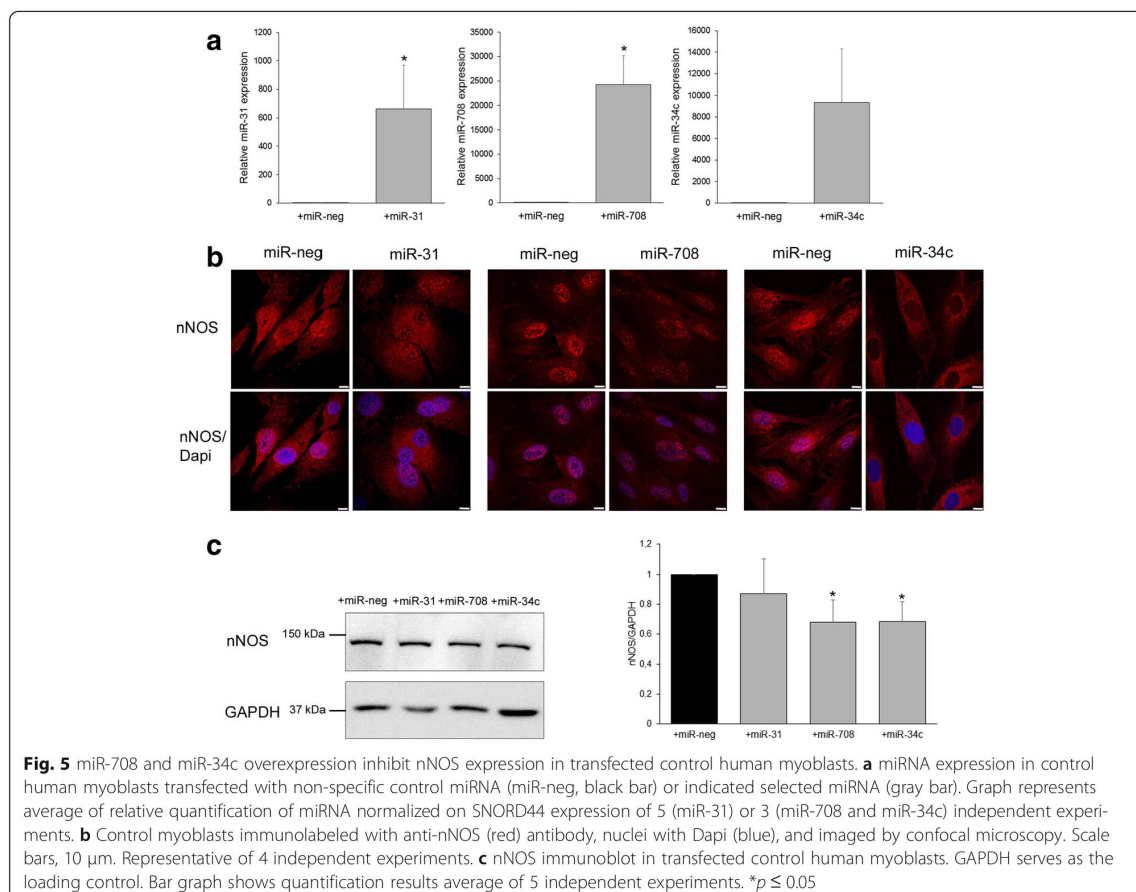
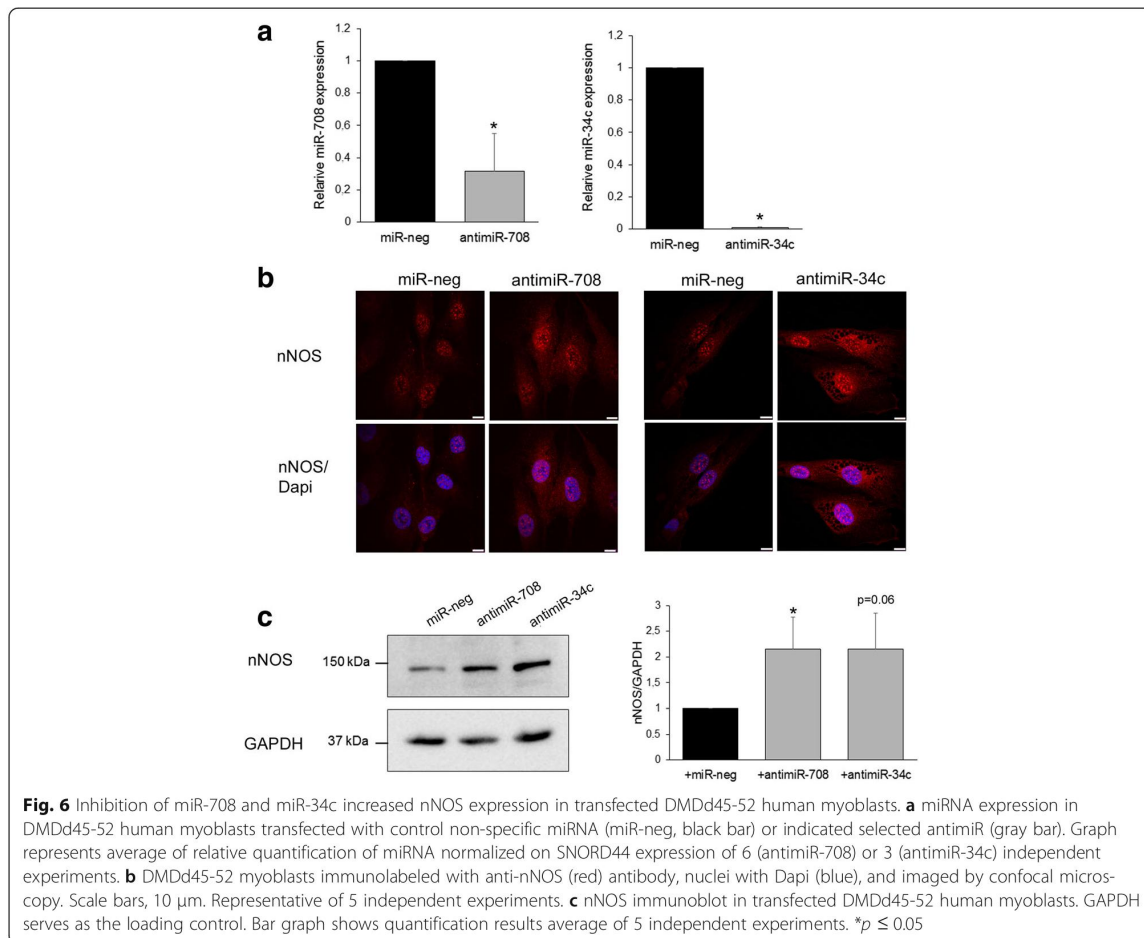


Fig. 5 miR-708 and miR-34c overexpression inhibit nNOS expression in transfected control human myoblasts. **a** miRNA expression in control human myoblasts transfected with non-specific control miRNA (miR-neg, black bar) or indicated selected miRNA (gray bar). Graph represents average of relative quantification of miRNA normalized on SNORD44 expression of 5 (miR-31) or 3 (miR-708 and miR-34c) independent experiments. **b** Control myoblasts immunolabeled with anti-nNOS (red) antibody, nuclei with Dapi (blue), and imaged by confocal microscopy. Scale bars, 10 μ m. Representative of 4 independent experiments. **c** nNOS immunoblot in transfected control human myoblasts. GAPDH serves as the loading control. Bar graph shows quantification results average of 5 independent experiments. * $p \leq 0.05$

in cell proliferation [39] and of porcine satellite cells by inhibiting Notch1 signal pathway that is involved in satellite cell quiescence [40].

The present study revealed that the nNOS expression could be modulated by miR-708 and miR-34c. Our results clearly showed their effect at the protein level, although we did not succeed to detect nNOS mRNA in myoblasts to demonstrate also the decay of its transcript. Interestingly, it should be noted that the isoform of nNOS that was detected in myoblasts by Western blot is about 140 kDa in size. In mature skeletal muscle, the nNOS μ , a 165-kDa protein, is the major isoform; it is linked to dystrophin via its PDZ domain [41] and thus located mainly at the sarcolemma (Additional file 4: Figure S1). However, this isoform seems too large to correspond to the nNOS isoform detected in myoblasts. Another isoform, the nNOS β which is 136 kDa in size, not displaying the PDZ domain [42], was described to be present in the Golgi apparatus of skeletal muscle fibers where it modulates the contractile apparatus [17] or at the sarcolemma of mice TA muscles [43]. Western blot

experiments on human muscular biopsy of healthy subjects showed a major 160 kDa in size nNOS isoform, as expected but also the 140-kDa isoform (Additional file 4: Figure S1). Additionally, we described here that the nNOS protein was localized in the nuclei of human myoblasts, as shown by immunostaining experiments. Western blots carried out on nuclear and cytoplasmic fractions confirmed that 140 kDa nNOS was detected in nuclei of control and DMD45-52 myoblasts (Additional file 4: Figure S1). Furthermore, a protein of about 160 kDa in size was only visible in nuclear extracts of both types of cells. These data were compared to those obtained from immunostaining experiments performed on DMD patient muscular biopsies which revealed nNOS expression in the nuclei of fibers of DMD muscle whereas nNOS is sarcolemmal in control muscle as expected (Additional file 4: Figure S1). Nuclear 160-kDa nNOS localization has been already described during C2C12 differentiation; however, authors of this study used a N-terminal nNOS antibody, that did not allow the detection of nNOS- β , and therefore a 140 kDa



nNOS isoform [44]. Our data suggest the presence of an isoform of nNOS not yet described in nuclei of myoblasts. At transcriptional level, the precise sequence of a transcript that encodes a nNOS of 140 kDa in size is not described in databases (i.e., Ensembl.org). The complexity of the mechanisms modulating *NOS1* transcription indicates that the nNOS isoform expressed in myoblasts and regulated by miR-34c and miR-708 has not been precisely identified and that information on the transcriptional regulation of its gene remains to be thorough.

The exact role of nNOS in nuclear compartment is still not well-defined. However, NO production has been designated as a key player which mediates epigenetic changes through the direct control of histone deacetylases (HDACs). Indeed, in the *mdx* mice defective for NO pathway, the activity of HDAC2 resulted to be specifically increased [45]. Profiling of human DMDd45-52 patient myoblasts confirmed the dysregulation of miR-1 but also found a significant

dysregulation in the expression of miR-29a, both of which regulate a dystrophin-nNOS-HDAC2 pathway [14]. In the present study, we could not exclude a link between nuclear nNOS location, HDAC2 nitrosylation, and the modulation of the miR-31, miR-708, and/or miR-34c expression. Nevertheless, a study in its own right will be necessary to establish this link.

Conclusions

Altogether, the present work highlights two miRNAs overexpressed in dystrophic human muscle as modulators of nNOS expression. This work could explain some pathological consequences caused by nNOS deficiency (i.e., muscle fatigability due to insufficient vasodilation in exercise, switch to glycolytic metabolism). In particular, modification of NOS1 expression has a significant negative impact on dystrophic muscle regenerative capacity [15], and it has been shown that treatment with NO donors can attenuate atrophy and metabolic changes and prevent changes in regulation [16]. We show here that

inhibitors of miR-708 and/or miR-34c could also be considered as therapeutic targets to rescue these defects by increasing the expression of nNOS. Furthermore, the expression and the sarcolemmal localization of the nNOS by interacting with the dystrophin has been shown to be crucial for contractile activity and muscular strength recovery in the canine DMD model (GRMD) [46]. Thus, a therapeutic strategy combining the inhibition of miR-708 and miR-34c with the restoration of dystrophin will most likely be a benefit for the improvement of phenotype of DMD and BMD patients.

Additional files

Additional file 1: Supplementary methods. (DOCX 12 kb)
Additional file 2: TLDA data. (XLSX 749 kb)
Additional file 3: Table S1. Predictive candidate miRNA binding sites on the human NOS1 3'UTR (DOCX 12 kb)
Additional file 4: Figure S1. Nuclear localization of nNOS in DMD muscular biopsy and in myoblasts. a) Control (ctrl) and DMD human muscular biopsy sections immunolabeled with anti-nNOS (red) antibody, nuclei with Dapi (blue), and imaged by confocal microscopy. Representative of 4 DMD patients. b) nNOS, GAPDH, and histone H3 (H3) immunoblots on cytoplasmic (CE) and nuclear (NE) protein extracts from control (ctrl) and DMDd45-52 myoblasts and total extract of control human muscular biopsy (ctrl biopsy). (TIFF 912 kb)

Abbreviations

BMD: Becker muscular dystrophy; DAPC: Dystrophin-associated protein complex; DMD: Duchenne muscular dystrophy; GMWS: Gardner–Medwin and Walton scale; GRMD: Golden Retriever muscular dystrophy; HDAC: Histone deacetylase; nNOS: Neuronal nitric oxide synthase; TLDA: Taqman Low-Density Array

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on a reasonable request.

Authors' contributions

FPR conceived the study. FPR and MG wrote the paper. MG made most of the experiments. LJJ performed the TLDA assays. KM provided the immortalized human myoblasts. FL and RBY provided the BMDd45-55 muscular biopsies and clinical information about patients. CGe, CP, EG, IH, CGr, and SF provided technical and helpful material. MG, FPR, CGe, SF, KM, FL, and RBY reviewed the final version of manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Muscle biopsies were collected from patients after informed consent, and this study was performed with agreement from the Committee for the Protection of Persons (CPP) concerned.

Competing interests

The authors declare that they have no competing interests.

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ARTICLE 4

Context:

Before I have begun my CIFRE/ANRT scholarship I was a sub-investigator in natural history and clinical trials phase 1, 2, 3 in DMD, SMA and myotubular myopathy (MTM) at the I-Motion platform of paediatric clinical trials. This experience motivated me to look for a translational project in order to start a PhD. I report here a published review based on my personal experience in AON treatment of SMA type 1 and 2 patients on the was proposal of the scientific committee of the French Society of Myology.

This report underlines a high phenotype and therapy response variability in SMA patients, which is in line with the manuscript subject.

Abstract:

Spinraza is the first FDA/EMA approved therapy in SMA type 1, 2, 3 patients. The first injection in EAP program was realized in France under the ATU program at the I-Motion platform of paediatric clinical trials. This review reports the overview of the literature focused on long-term efficacy results in SMA type 1 and 2 patients and reveals a clinically heterogeneous population with a great variability in therapy response. Overall, relatively long-term results confirm the initial data that early therapy initiation is correlated to better response.

► Une révolution est actuellement en cours dans le domaine des maladies neuromusculaires avec l'arrivée de nouvelles thérapies. L'amyotrophie spinale (SMA ou *spinal muscular atrophy*) est parmi les maladies pionnières de ce bouleversement thérapeutique. Le premier traitement approuvé et mis sur le marché en Europe et aux États-Unis est un oligonucléotide antisens dénommé nusinersen et commercialisé par le laboratoire Biogen sous le nom de Spinraza®. Il a comme indication les SMA de types 1, 2 et 3. La première injection de Spinraza® dans le cadre d'une ATU/EAP (Autorisation Temporaire d'Utilisation/Expanded Access Program ou programmes d'accès étendu) a été réalisée en France par le centre d'essais I-Motion. Les résultats des essais cliniques et des données de la littérature sur l'utilisation du nusinersen dans la SMA infantile sont discutés dans cette revue. Ces études rapportent une amélioration de la fonction motrice chez les patients SMA tous types confondus y compris les patients de type 3 [1,2]. Une administration précoce du traitement s'accompagne d'une meilleure réponse clinique. Une meilleure compréhension de l'hétérogénéité génétique et clinique devient indispensable dans le monitoring et le suivi à long terme de ces patients. ◀

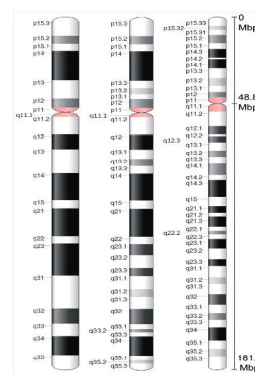
État des lieux

La SMA est une des maladies neuromusculaires les plus fréquentes de l'enfant mais concerne également les adultes. C'est la cause génétique la plus fréquente de mortalité infantile [3]. Selon l'âge du début des premiers signes et le niveau moteur maximal atteint, la SMA est classée selon cinq types. La SMA de type 0 avec un début des signes dans la période anté- ou néonatale. La SMA de type 1 (ou maladie du Werdnig-Hoffmann) se caractérise par un début des premiers signes

Les oligonucléotides anti-sens dans la SMA

Retour d'expérience et données de la littérature

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avant l'âge de 6 mois et une durée de vie sans traitement inférieure à 9-18 mois. Certains patients, dits de type 1bis, survivent plus de 18 mois mais n'acquiescent jamais la station assise indépendante. Pour la SMA de type 2, les premiers signes apparaissent après 6 mois et les enfants sont capables de se tenir assis de façon indépendante pendant plus d'une minute. Pour la SMA de type 3, (ou maladie de Kugelberg-Welander) le début est plus tardif, avec un risque important de perte progressive d'une marche autonome initialement acquise. Enfin la SMA de type 4 est caractérisée par un début des symptômes à l'âge adulte [4].

La SMA a une incidence de l'ordre de 1/10 000 naissances avec une fréquence de porteurs sains hétérozygotes de 1/50 [5]. C'est une maladie autosomique récessive due à des mutations dans le gène *SMN1* (*survival motor neuron 1*) localisé sur le chromosome 5q13.2 [6]. Elle se caractérise par une faiblesse musculaire proximale suite à la dégénérescence progressive des motoneurons de la corne antérieure de la moelle épinière et du tronc cérébral. La prévalence communément admise pour la France (enquête SMA-Flash, Filmemus) tourne autour de 1200 patients SMA dont 8 % de type 1, 47 % de type 2, 42 % de type 3 et 2 % de type 4 [7].

Données génétiques dans la SMA

Dans 95 % des cas environ, la SMA est due à une délétion homozygote, soit de l'exon 7, soit des exons 7 et 8, du gène *SMN1* (5q12.2-q13.3) codant la protéine de survie du motoneurone (SMN). Situé en région



centromérique, le gène *SMN2*, est capable de produire la protéine SMN, mais celle-ci est non fonctionnelle dans 90 % des cas à cause d'une erreur d'épissage. La seule différence entre les deux gènes réside dans la substitution C→T qui génère un épissage alternatif de l'exon 7. Les 10 % de la protéine SMN produite par le gène *SMN2* sont insuffisants pour maintenir la survie et le fonctionnement normal des motoneurons [6]. Le rôle de la protéine SMN n'est pas encore complètement connu. Elle est associée avec la biogenèse de la protéine nucléaire ribonucléique (snRNP) et le métabolisme des ARN messagers [8]. De plus, les données de la littérature suggèrent qu'en son absence survient une dégénérescence des neurones sensitifs et de la jonction neuromusculaire [8].

Hétérogénéité phénotypique des patients atteints de SMA

Le nombre de copies du gène *SMN2* est inversement corrélé avec le phénotype clinique. Il est important de souligner que le nombre de transcrits *SMN2* n'est pas forcément identique au nombre de copies de *SMN2* à cause de la variabilité de la régulation de la transcription inter individus. Des données récentes ont rapporté des polymorphismes du *SMN2* en tant que modificateurs géniques [9 -11]. Malgré cela, aux États-Unis, le groupe de travail multidisciplinaire *SMA NBS* a émis les recommandations d'algorithme thérapeutique basé sur le nombre des copies de *SMN2* pour les patients SMA diagnostiqués par dépistage néonatal [12]. Il est important de connaître et de comprendre au mieux l'hétérogénéité génique et clinique des patients pour le diagnostic et le pronostic clinique mais également pour comprendre et quantifier la réponse thérapeutique.

Options thérapeutiques

Diverses thérapies incluant des oligonucléotides antisens (ASO) et des petites molécules ont été mises au point pour interférer avec l'épis-

sage alternatif de *SMN2* augmentant la transcription de l'ARN messenger et par voie de conséquence de la protéine SMN produite. Parmi ces nouvelles thérapies, Spinraza® (nom commercial du nusinersen) s'administre en injections intrathécales, avec une dose fixe de 12 mg par administration pour tous les patients tout âge confondu [1, 2]. Le traitement doit débiter le plus tôt possible après le diagnostic, avec quatre doses de charge aux jours 0, 14, 28 et 63. Une dose d'entretien doit ensuite être administrée tous les quatre mois.

D'autres options thérapeutiques telles que l'injection unique d'AAV par voie intraveineuse pour traiter les enfants SMA de moins de deux ans (Zolgensma®) et les petites molécules (comme la flunarizine) ne seront pas abordées dans cette revue.

Les essais cliniques nusinersen dans la SMA

Les résultats précliniques encourageants et des études en ouvert ont permis la réalisation des essais cliniques de phase 3 pour évaluer l'efficacité thérapeutique du nusinersen chez les patients SMA de types 1 et 2 (Tableau I).

En Europe, le nusinersen a été administré initialement dans le cadre d'un programme d'autorisation temporaire d'utilisation (ATU/EAP). Certains résultats du suivi de ces patients seront discutés ci-dessous.

SMA de type 1

Dans l'essai clinique multicentrique ENDEAR (CS3B) de phase 3 contrôlé en double aveugle ont été inclus

	CS3A	Endear (CS3B)	CS2/CS12	Cherish CS4	Nurture
Sponsor	Ionis	Biogen	Ionis	Biogen	Biogen
Design de l'étude	En ouvert	Contrôlé	En ouvert	Control vs procédure placebo	Contrôlé ou en ouvert
N	20	123	28-58	126	17
Critères d'inclusion	<7 mois SMA1	<7 mois SMN2=2 SMA1	2-15 ans SMA 2,3	2-12 ans SMA2	Présymptomatique Exam neuro NL CMAP1 mV
Evaluations	Survie/resp. HINE2 CHOP INTEND CMAP	Survie/resp. HINE2 CHOP INTEND CMAP	HFMSE 6MWT RULM	HFMSE RULM	Survie/resp. HINE2 CMAP

Tableau I. Essais cliniques avec traitement par Spinraza® (Nusinersen) (adapté de Claudia A. Chiriboga [13]). AS : amyotrophie spinale ; N : nombre des patients ; NL : normal ; exam neuro : examen neurologique ; resp. : ventilation respiratoire permanente, trachéostomie ou ≥ 16 h de ventilation ; HINE2 : The Hammersmith Infant Neurological Examination ; CHOP INTEND : The Children's Hospital of Philadelphia Infant test for Neuromuscular Disorders ; HFMSE : Hammersmith Functional Motor Scale Expanded ; 6MWT : test de marche de 6 min ; RULM : Revised Upper Limb Module ; CMAP : Compound Muscle Action Potential.



121 enfants avec SMA de type 1, tous avec deux copies de *SMN2* et âgés de moins de 7 mois à la visite de sélection. L'étude a été arrêtée prématurément au bout de 13 mois suite aux résultats d'analyse intermédiaire qui ont montré une réponse positive significative du test HINE (*Hammersmith Infant Neurological Examination*) chez les patients sous traitement. Les patients ont été tous inclus dans le groupe traité et à la fin de l'étude les survivants ont été inclus dans l'étude CS11 [1].

SMA de type 2

Dans l'essai clinique CHERISH (CS4) de phase 3 multicentrique, contrôlé en double aveugle, 126 enfants ont été inclus. Les patients inclus présentaient une SMA de type 2 (2 à 12 ans), sans scoliose sévère ou rétractions articulaires avec une randomisation de 2:1. L'analyse intermédiaire à 15 mois a mis en évidence une différence significative du score HFMSSE (*Hammersmith Functional Motor Scale Expanded*) entre le groupe traité versus contrôle, ce qui a conduit à un arrêt prématuré de l'étude et la mise en place d'une phase d'extension CS11 [2].

Patients présymptomatiques

Dans l'étude NURTURE (CS5/SM201), 17 patients ont été inclus avec diagnostic prénatal ou à la naissance de SMA. La majorité des patients avait deux copies de *SMN2* (12/17). Tous les patients traités ont survécu sans aide ventilatoire et ils continuent de progresser dans leur développement psychomoteur.

Patients SMA de type 1/1bis tout âge confondu traités sous ATU

Plusieurs études récentes ont évalué l'évolution clinique chez des patients SMA de types 1 et 1bis traités par Spinraza® dans le cadre de l'ATU (EAP) [13, 14]. En comparaison avec les essais cliniques, les patients inclus dans ces cohortes étaient d'âges variables et avec une durée d'évolution de la maladie plus longue et un début de traitement parfois bien au-delà de la limite des 6 mois choisie lors des essais cliniques. Globalement, les évaluations motrices chez les patients avec un début de traitement précoce avant 7 mois ont été comparables aux résultats rapportés dans les essais cliniques. L'étude italienne rapporte une amélioration après 12 mois de traitement des résultats de CHOP INTEND (*Children's Hospital of Philadelphia Infant Test Neuromuscular Disorder scale*) (moyenne 5,46 points) et dans moins de 15 % des cas une aggravation dans cette cohorte [9]. Dans une étude allemande, il a été proposé aux parents de donner leur appréciation sur l'évolution sur le plan moteur chez les enfants SMA de type 1bis traités. Dans 93,4 % de cas, la réponse au traitement a été jugée favorable par les familles. Cette appréciation n'était toutefois pas significativement corrélée avec les résultats du CHOP-INTEND [13]. Par ailleurs, il est encore difficile d'établir une corrélation des progrès identifiés avec la CHOP-INTEND et leur traduction au quotidien chez les patients avec SMA de type 1. Cette échelle reste néanmoins un outil précieux de suivi des patients SMA. Après la mise sur le marché du nusinersen, la question de poursuivre ce type de traitement lourd et onéreux se pose pour les patients avec peu ou avec des progrès modestes. Dans

l'étude italienne, moins de 7,5 % des patients ont arrêté le traitement [13]. Le suivi des patients, issus de différents pays de la Communauté Européenne, a mis en évidence une différence de prise en charge entre centres et entre pays.

Conclusion

Spinraza® (nusinersen) est le premier traitement approuvé et mis sur le marché en Europe et aux États-Unis. La première injection de Spinraza® dans le cadre d'une ATU a été réalisée en France (Institut I-Motion, Hôpital Trousseau). Le traitement des patients SMA de type 1 est maintenant devenu possible dans de nombreux centres hospitaliers en France. Spinraza® est aussi disponible actuellement pour les patients SMA de types 2 et 3. L'intérêt du traitement serait de préserver les acquisitions des patients et d'empêcher la progression de la maladie. Chez les patients avec la SMA de type 1bis, l'indication au cas par cas semblerait être une attitude plus prudente suite aux résultats modestes chez ces patients dans les cohortes traitées initialement dans le cadre d'une ATU. Les données précliniques et les résultats des essais thérapeutiques mettent en évidence l'existence d'une fenêtre d'administration thérapeutique pour une meilleure réponse clinique. Ces données ouvrent le débat autour du dépistage néonatal pour la mise en place du traitement dans les délais recommandés par les essais cliniques. Un groupe de travail *ad hoc* est désormais en place sous l'égide de la filière FILNEMUS rassemblant les professionnels de santé concernés et les représentants de patients. Les études publiées sur les cohortes des patients traités sous ATU en Europe ont mis en évidence une large hétérogénéité dans la prise en charge de ce type de

Leçons apprises

- Diagnostic urgent avec un choix de traitement disponible.
- Nécessité d'accompagnement des familles devant ce choix cornélien.
- Nécessité d'organisation locale pour la mise en place précoce du traitement.
- Création d'un registre pour la surveillance de l'évolution des patients traités.
- Plusieurs choix thérapeutiques bientôt disponibles.
- Nécessité d'ouvrir le débat autour du dépistage néonatal de la SMA.
- Implémentation des standards de soins.
- Hétérogénéité génique et clinique des patients.

patients, avec la présence dans certains cas de l'assistance ventilatoire et/ou des différences dans le type de rééducation motrice et la prise en charge nutritionnelle. Ces études soulignent largement l'intérêt de l'implémentation des standards de soins concomitante à toute démarche thérapeutique. ♦

Antisense oligonucleotides in SMA: lessons learned and literature data

LIENS D'INTÉRÊT

E. Gargaun a travaillé en tant qu'investigateur dans les essais cliniques sur le nusinersen et a réalisé les premières injections dans le cadre de l'ATU en France à l-Motion.

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TIRÉS À PART

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CHAPTER III: DISCUSSION

CLINICAL CHARACTERIZATION

This thesis project was initiated based on clinical observation of phenotype heterogeneity in patients with similar genetic pattern. Phenotypic and genotypic characterization of BMDdel45-55 patients can reveal the potential therapy efficacy of MES 45-55. Thus, detailed characterization of this population was very timely and of great importance considering the rapid advancement of development of therapy strategies and personalized screening.

SKELETAL MUSCLE CHARACTERIZATION ACCORDING TO THE CLINICAL SEVERITY SCALE (CSS)

Here we present the clinical retrospective characterization of the largest BMDdel45-55 patients. Despite the retrospective character of this study, main clinical features and precisely the skeletal muscle and cardiac involvement has been described in 50 patients. Skeletal muscle symptoms and deficit were characterized by a new in-house Clinical Severity Scale (CSS). Phenotype distribution in this cohort was reported according CSS type and in 100% patients featured BMD phenotype with in the large majority mild (MBMD) and intermediate (IBMD) type and only one asymptomatic (ABMD) patients and two severe (SBMD). Compared to reported data in literature we described a more heterogeneous phenotype than Taglia et al., indeed in their study authors reported 9 BMDel45-55 patients with in large majority except one asymptomatic diagnosis. There is no evidence form the reported clinical feature in favor of skeletal muscle deficit and the muscular involvement was apparently limited to calf and quadriceps hypertrophy⁶⁹. Six asymptomatic patients were reported in a different study with high CK as predominant cause of diagnosis. Interestingly, one additional patient had skeletal muscle deficit and lost ambulation at 79 years¹⁹⁷. This data is in line with our report as we showed a slowly progressive evolution and only 2 patients with loss of ambulation. Interestingly, retrospective CSS scoring in SBMD and IBMD allowed us to show the progressive character of the disease

by identification of the age at reaching intermediate and severe stage. In addition, the duration of the disease evolution free of skeletal muscle deficit and preserved ambulation was reported and reflect the quality of life of BMDdel45-55 patients.

Also, based on the slowly progressive character of disease in this specific mutation, we were able to reveal that there are two different profiles in this population. Indeed, there is a small number of patients that have a trend to remain mild and thus will remain free of skeletal muscle deficit. In the second one, patients will feature walking and running difficulties and considering the advanced age of loss of ambulation, these patients are still at risk to have partial of total wheelchair bound after the age of 60 years. Based on this data and despite a favorable phenotype of BMDdel45-55 patients we confirm the heterogeneous phenotype of skeletal muscle features.

Indeed, previous reports from our team described clinical and histological characteristic in 12 patients with BMDdel45-55⁷¹. The clinical status was based on Gardner-Medwin and Walton scales and included data of disease progression as several patients had reports of 20 years follow-up. CSS scoring classified as IBMD the same patients identified as with moderate phenotype in the first study. In addition, the histological status based on hematoxylin and eosin (HE) staining in the patients with IBMD revealed fiber size heterogeneity and advanced dystrophic features with adipocytes and fibrosis similar to DMD samples. **This data illustrates a good correlation of CSS scale with the clinical and histopathological conditions in BMDdel45-55.** Moreover, dystrophin quantification in the same patients showed variability in the relative quantification with values ranging from 50% to 90%.

While it is important to underline that muscular biopsy data should be interpreted taking into consideration the age of realization, the muscle type but also the delay from the disease first skeletal muscle symptoms, this data implies that patients with similar clinical phenotype can have different amount of dystrophin protein. This imply that therapy development targeting dystrophin restoration should consider additional functional outcome measures in addition to dystrophin relative quantification.

CARDIAC IMPAIRMENT

In addition to skeletal muscle phenotype we reported a heterogeneity in cardiac impairment. Indeed, in this work we highlight that BMDdel45-55 has a high percentage of cardiomyopathy in 33 % of patients and one patient who underwent cardiac transplant. Compared to literature data patients with del48 in DMD gene is reported to have 20% of CDM (n=10) (Bello et al 2016). Also, in this work we reported a later age of onset of CMD (mean 46 years). Recent literature data reported that disruption of spectrin repeat phasing as in *DMD* exon 45-49 deletion is associated with significantly earlier onset of DCM^{204,205}.

Most interestingly, based on the age of the LVEF examination and the CSS phenotype, we report three distinct groups of patients, the first one is the young patients without CDM with MBMD and ABMD, the second adults without CDM (IBMD) and the third one the adults with CDM (IBMD and SBMD). In addition, one patient undergone cardiac transplant despite a mild clinical phenotype.

Altogether, the clinical characterization allowed us to confirm the BMD phenotype in 100% and reveal a skeletal muscle and cardiac heterogeneity within this cohort of patients. This data confirms the favorable outcome of MES45-55 therapy development. Also, patient stratification should be considered by CSS type and cardiac impairment.

GENOTYPIC CHARACTERIZATION

WGS ANALYSIS

In order to address the question of the clinical heterogeneity in this specific BMDdel45-55 populations we performed WGS analysis in 19 patients. We searched for SNPs potentially associated with skeletal muscle and cardiac phenotype. Except for one SNP in one patient we did not found any potential cardiac SNP. Surprisingly, in family cases, we did not identify any common variants regardless of the type of analysis. Our results are in line with literature data, patients with early signs of cardiomyopathy and long survivor patients without significant

cardiac involvement shared common variants (rs 1060575, rs 3816989). Moreover, the reported variants associated cardiac involvement and loss of ambulation suggesting that identified gene and variants could involve both cardiac and skeletal muscle²⁰¹.

Taking into consideration the limited amount of WGS data, we looked closely in the characterization of two patients with SBMD according to CSS who displayed additionally severe cardiomyopathy. Interestingly these patients did not have similar disease progression, except for the age of first clinical signs reported in early childhood. In patient 11, Severe BMD and with severe cardiomyopathy we report a SNP in *FLNC* gene identified as a potential candidate for future studies. Based on a recent study on Dp116 pathogenic role in cardiomyopathy we searched the WGS data in BMDdel45-55 patients in order to identify if the exon 1 of Dp 116 localized in intron 55 was conserved on genomic level ^{206,207}. One of the explanations of the cardiac phenotype variability could be the promoter activation of the Dp116 localized in the intron 55. In a recent study authors reveal association for the Dp116 with cardiomyopathy in DMD. Indeed, one of the SBMD patient, N11 conserved the exon 1 of Dp116 isophorme and patient 19 did not. Interestingly, patient N11 had a rapid decline of skeletal muscle deficit compared to patient 19. Unfortunately, we did not had access to muscle biopsy in order to confirm the expression of Dp116 isoform in these two patients. Interestingly, the same patients N11 with the identified potential pathogenic SNP in *FLNC* gene association conserved also the exon1 of Dp116 localized in intron 55. Ongoing gene modifier detection in WGS data of BMDdel45-55 patients will allow to complete this personalized profiling.

We reported the results of SNPs analysis in cardiac gene responsible for major cardiac impairment and highlighted the absence of any potential association of reported SNPs with pathogenic potential in 19 patients with BMDdel45-55. Identification of patients with extreme clinical conditions according to CSS scale and cardiac impairment revealed one SBMD patients with association of SNP in *FLNC* gene and genomic presence of exon1 Dp116 isophorme that could possible participate to the severity of his phenotype. Additional experiments are necessary to demonstrate this hypothesis. Altogether, these data suggest a favorable outcome for MES45-55 and demonstrate WGS utility in patient stratification.

BREAKPOINT ANALYSIS

In addition to the SNP and gene modifiers profiling we analyzed the breakpoint in 19 WGS patients. In result, we determined that except for family cases, each patient had his singular breakpoint (+20bp) (figure 1 in article 2). Previously reported breakpoint analysis in 3 BMDdel45-55 patients did not revealed any substantial homologies between the normal DNA and in one case, the breakpoint was located in LINE-1 repetitive element. We identified the all repetitive elements form intron 44 and 55 (data not shown).

GENOMIC PROFILE AND CHARACTERIZATION OF LNCRNA LOCALIZED IN INTRON 44 AND 55

Furthermore, the breakpoint analysis in 19 patients identified different clusters of genomic presence in four lncRNA sequences boarding exon deletion 45-55 (lncRNA 44s, 44s2, 55s and 55as). We completed WGS data with PCR sequence detection in 38 BMDdel45-55 patients. Interestingly, phenotype correlation allowed us to reveal favorable phenotype in patients preserving lncRNA44s localized in intron 44. Indeed, our in vitro experiment data in human primary and immortalized myoblasts reveal accelerated differentiation. lncRNA 44s2 over expressed increase *MyoD* and *MyHC* expression at 3 days of differentiation. Moreover, in muscle biopsies study from BMDdel45-55 patients the lncRNA expression was similar to control suggesting active regeneration process. Interestingly, the lncRNA expression was very weak in muscular biopsies of DMD and BMDdel3-5 and del3-7 patients. This data confirms phenotype variability among BMD patients and suggests that lncRNA expression could be associated with phenotype severity and participate to phenotype variability.

In contrary to the literature data, we did not confirm the negative regulatory role of lncRNA on pre-mRNA of dystrophin. One of the explanations can be the fact that the lncRNA (44s, 44S2 and 55s) over expression study of Bovolenta et al was conducted in human rhabdomyosarcoma

and neuroblastoma cells. It could be interesting to conduct lncRNA44S2 over expression at different time-points of differentiation and study the dystrophin mRNA expression consequently.

Altogether these data identify the lncRNA 44s2 as a potential biomarker of DMD and BMD disease progression. Literature data described in the introduction section reported the potential of lncRNA as therapeutic, disease progression biomarkers. Indeed, based on our hypothesis that lncRNA44s2 reflects the regeneration potential of skeletal muscle, absent expression in the severe DMD phenotype could be monitored during therapy clinical trials and indicate a potential efficacy of a drug by inverting this expression pattern.

CONCLUSION

Indeed, we hypothesized that lncRNA 44s and 44s2 expression could reflect the regeneration activity. Figure 3 from article 2 illustrates the expression pattern of lncRNA in 3 different type of patients. The phenotypic spectrum of these patients could be interpreted as reflecting the severity spectrum of the muscular dystrophy.

First, healthy control biopsy could reflect physiologic myogenesis in adult skeletal muscle. Following, BMDdel45-55 patients muscle biopsy is supposed to illustrate a favorable clinical phenotype with ongoing active regeneration. Finally, we depicted this theoretical expression distribution in the following Figure 17. According to our hypothesis, controls (WT) and DMD patients have similar patient severity distribution (Figure 17 right axis, in red) and BMD patients are more heterogeneous. As for lncRNA expression (Figure 17 left axis, in blue) based on reported results from expression assessment in skeletal muscle biopsies, the DMD patients represent the lowest level of expression corresponding to the severity of the disease with severe DMD patients with absent regeneration.

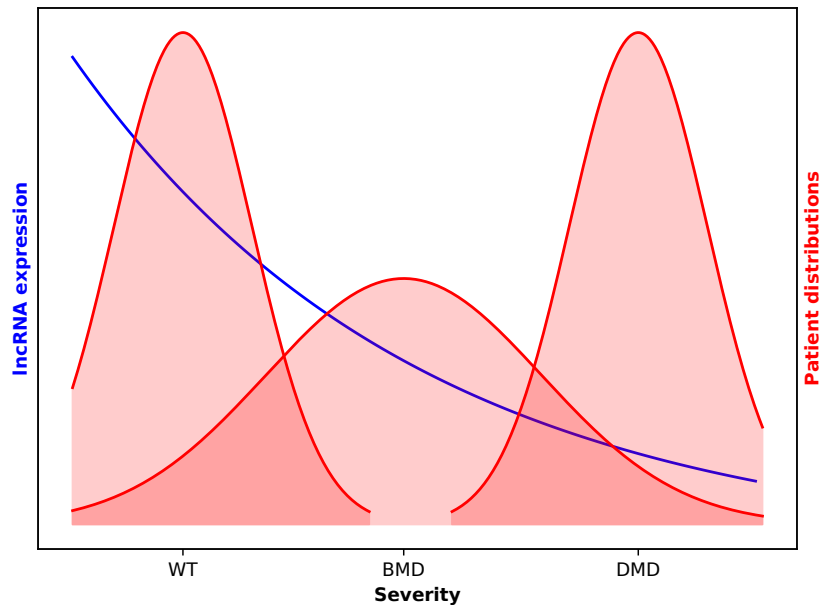


Figure 20 Theoretical lncRNA expression in control, BMD and DMD patients.

Our results reflect lncRNA expression in DMD myoblast at three days of differentiation but do not find lncRNA expression in muscular biopsies from DMD subjects. There are several possible explanations, the first one is that human primary myoblasts could preserve some basal level of regeneration activity. Indeed, we reported a low level of MyoD expression at three days of proliferation, with an increase in MyoD expression only at 3 days of differentiation. This data suggests a delayed differentiation in human primary myoblasts from DMDdel45-52 patient (see MyoD expression in figure 4 from article 2). In contrary, the skeletal muscle biopsy reflects an advanced stage of skeletal muscle dystrophy with low level lncRNA expression. Secondly, the absence of dystrophin protein could participate to the low level of lncRNA expression. Indeed, BMDdel45-55 patients display a truncated but functional dystrophin in opposite to the DMD patients that do not express dystrophin at all. We can hypothesize that dystrophin presence could trigger lncRNA expression as a regulatory expression loop illustrated in Figure 18 below. Additional functional data of reported lncRNA could explore these theoretical hypotheses.

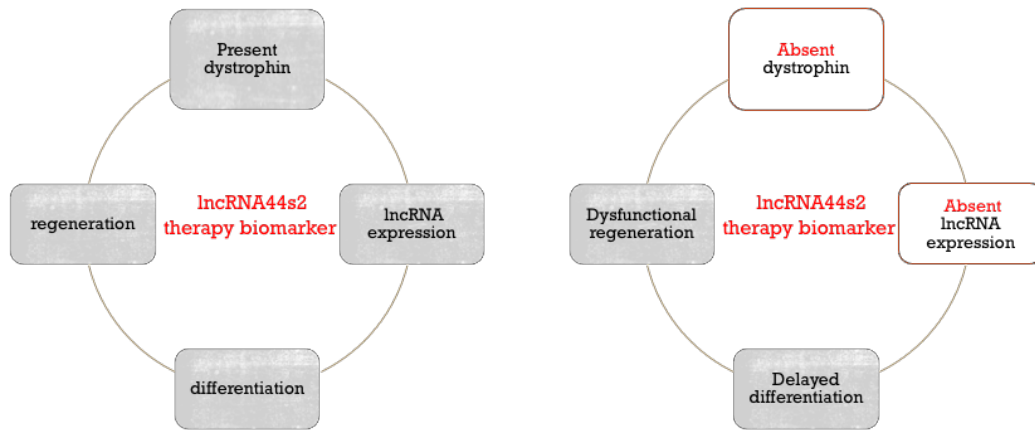


Figure 2 | lncRNA hypothetical regulatory loop in BMD and DMD.

In closing, this work allows a detailed phenotypic and genotypic characterization of the largest BMDdel45-55 cohort and confirm the favorable MES 45-55 prognostic. Moreover, the clinical heterogeneity in this specific cohort underlines the importance of the WGS and CSS stratification for future clinical trials.

CHAPTER IV: CONCLUSION AND PERSPECTIVES

RETROSPECTIVE CLINICAL CHARACTERIZATION OF BMDDEL45-55 BY A CLINICAL SEVERITY SCALE (CSS). PROSPECTIVE FEASIBILITY AND APPLICATION IN CLINICAL TRIALS?

In this work we studied phenotypic and genotypic characterization of BMDdel45-55 patients. Clinical characterization was retrospectively reported by assessing an in-house CSS. This severity scale allows to identify the progressive evolution of the muscular dystrophy. We demonstrated an age dependent correlation of CSS types suggesting a progressive course of the disease. In addition, the interrater variability was very low and inter-operator reproducibility was estimated at 100%.

Currently limited number of clinical trials are ongoing in BMD. One of the explanations of this is the lack of clear outcome for therapy efficacy monitoring. Moreover, in opposite to DMD clinical trials where dystrophin restoration is a major indicator of therapy efficacy, in BMD there is no clearly identified biomarker of disease monitoring nor therapy efficacy.

Based on the example of clinical trials in Spinal Muscular Atrophy (SMA), a clinical severity scale was used in addition to the genetic pattern in order to better stratify patients in phase 3 randomized clinical trials versus placebo. Indeed, treated infants with this lethal disorder survived but also switched from type 1 to type 2 demonstrating gain in quality of life.

Currently, several different scales are used in order to address the question of skeletal muscle deficit but their assessment require time and previous training.

Our proposed CSS has the advantage of responding to the main question of any consultation or clinical trial: the presence of absence of skeletal muscle deficit or loss of ambulation. In our opinion, based on this simple scoring of severity degree clinical meaningful improvement could be applied even to heterogeneous population as BMD patients.

WGS UTILITY IN PATIENT FOLLOW-UP, THERAPY DEVELOPMENT AND
IN CLINICAL TRIALS?

As demonstrated in this work, patient stratification according to a clinical severity scale and by performing WGS allowed to identify several patients with extreme clinical features. Indeed, from 100 % BMD cohort only two patients lost ambulation at 60 and 72 years old and both presenting severe cardiomyopathy. Interestingly, the breakpoint analysis allowed to identify the preservation of exon1 Dp116 that was described as associated with severe cardiomyopathy in DMD patients and in addition a potential pathogenic SNP association in FLNC gene. Ongoing study will allow to interrogate the presence of gene modifiers in this cohort. Altogether, this study highlights the utility of WGS in patient follow-up by better monitoring cardiac impairment.

Moreover, the detailed characterization of breakpoint in these patients allowed us to identify a lncRNA genomic cluster associated with a favorable clinical phenotype. This characterization could be useful for the MES development by CRISPC-Cas9 assay. Based on literature data CRISPC-Cas9 gRNA in actual development are localized closely to exons 44 and 56. In this report we identify only one patient with a large deletion excluding all lncRNA form intron 44 and 55. Based on these results, we can suggest that preservation of lnc44s2 could be beneficial in the development of a potential CRISPC-Cas9 gRNA therapy. Further experiments are necessary in order to validate this assumption and interrogate the role of lncRNA of interest.

IMPLICATION OF LNCRNA 44S, 55S AND 55AS IN THE REGENERATION PROCESS AND THE DYSTROPHIN EXPRESSION REGULATION?

In this work, we analyzed the lncRNA44s2 function by an OE experiment in human primary and immortalized myoblasts. We demonstrated the implication of lncRNA44s2 in differentiation in Myo45-55 cell line. However, we did not confirm previously reported negative regulation of mRNA dystrophin by OE of lncRNA44s2. One of the explanations could be the difference in the cell type used, the necessity to have more cell lines with same genetic pattern and perform longer experiments. Unfortunately, it was not possible during this work to access to primary myoblasts issued from different patients but with similar genetic mutation, of healthy control, BMDdel45-55 nor DMD45-52. However, we managed to use immortalized and primary myoblasts from the same patients for our experiments and confirm the reported results in both type of cell lines.

During my first year of PhD I have tried to create genetically modified cell lines using CRISPR-Cas9 gRNA in order to reproduce the identified genomic lncRNA clusters in BMDdel45-55 patients. Unfortunately, due to technical difficulties I had to abandon this approach.

In the figure below is depicted the initial strategy that was to transfect (Tx) directly the CRISPR-Cas9 sgRNA in immortalized myoblasts from healthy control. We used two pairs of sgRNA construct and despite a better Tx efficiency with the Tx110+117 sgRNA (Figure 23A) we did not obtain an efficient genomic modification of the transduced myoblasts.

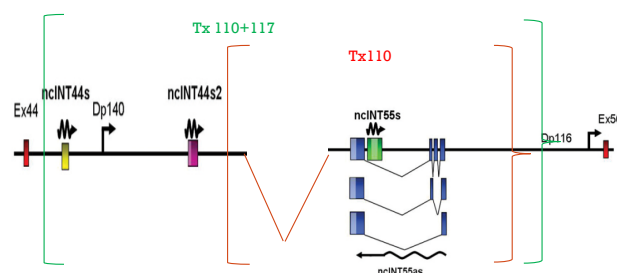


Figure 22 Scheme illustration of CRISPR sgRNA genomic localization. In green are depicted the couple of sgRNA Tx110+117 and in red the second used couple Tx110.

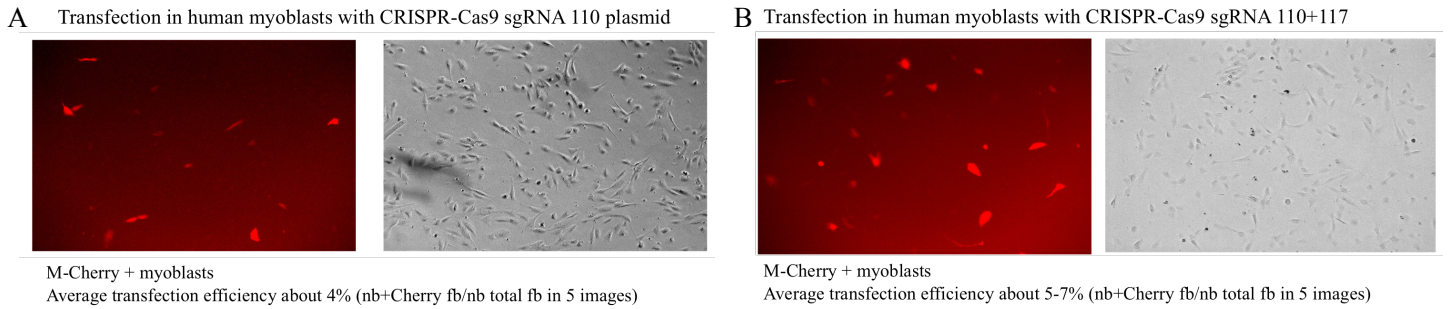


Figure 23 Human myoblasts CRISPR sgRNA transfection (Tx) assay. (A) M-Cherry positive fibers count after Tx with sgRNA 110 and 117. (B) M-Cherry positive fibers count after Tx with sgRNA 110.

In addition, we have selected the M-Cherry + cells by FACS (Figure 24). Despite the selection and a second Tx we did not reached a sufficient number of genomic modified myoblasts that were verified by RT-PCR with a nested “poison PCR” (Figure 25).

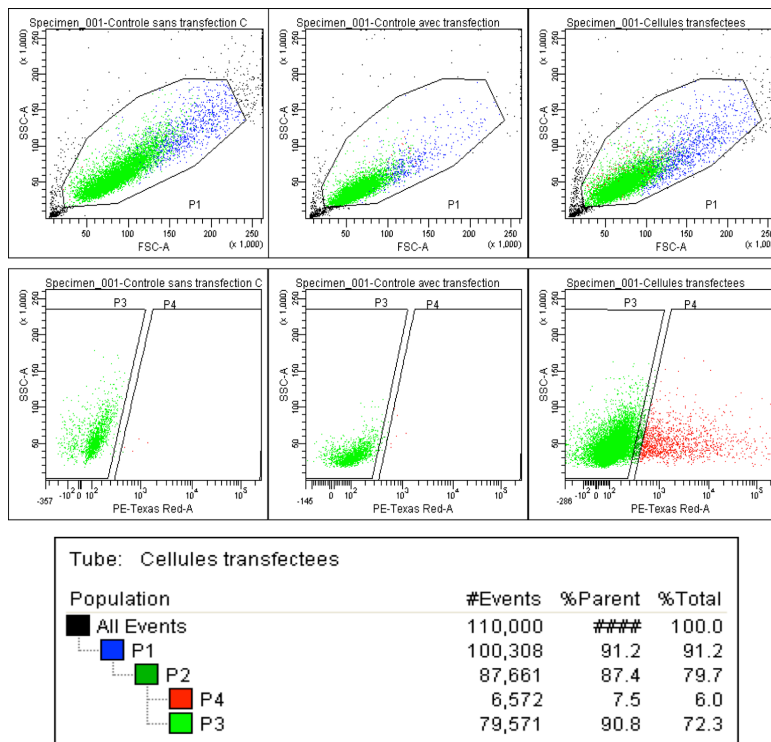


Figure 24 FACS selection. Detection of M-Cherry + cells by cell size and complexity by forward (FSS) and side scatter areas (SCC) gating with two controls (scramble transfected cells and normal human myoblasts).

mRNA DYSdel45-55 expression

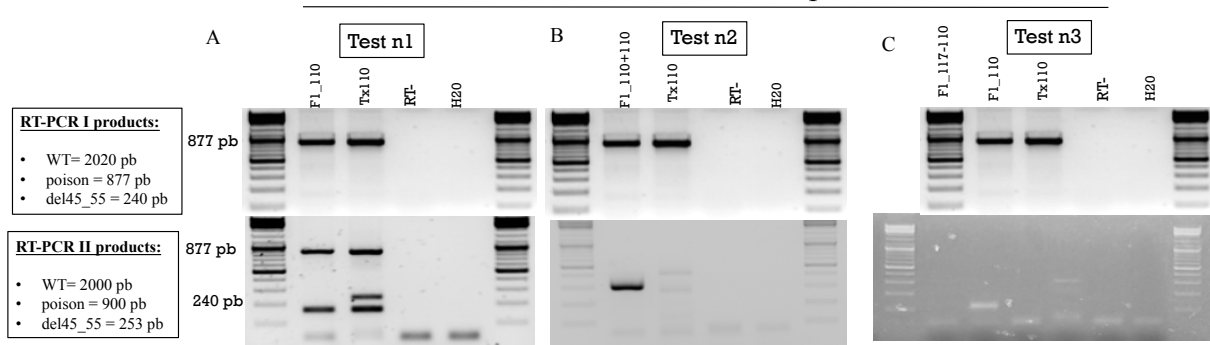


Figure 25 mRNA DYSdel45-55 detection by "poison" nested RT-PCR. (A) Test n1 illustrates the first strategy in order to improve the deletion efficiency by FACS sorting (B) by double Tx of same sgRNA and (C) by combined transfection. Despite all the attempted strategies sequencing validation failed.

Currently, a collaborative project with Matteo Bovolenta is ongoing with Génethon platform of CRISPR-Cas9 cell lines. In this project I have designed several genetic profiles for the future cell lines according with the profiles of lncRNA clusters. We have already tested and validated the CRISPR-Cas9 gRNA that are boarding the lncRNA44s2 in order to designs a KO model.

We hope that this project will provide experimental validation of different combined or single lncRNA KO.

In conclusion, given the rapid progress of MES 45-55 therapy development it is crucial to have precise prediction of the potentially resulting phenotype. In this work, we demonstrated the necessity of deep phenotyping and NGS in order to improve actual data in a strategic population of BMD patients by combining detailed phenotype description with WGS data for patient stratification and identification of potential phenotype modifiers. We hope that this work will help to demonstrated the utility of genetic sequencing and precise phenotypic characterization in the day-to day healthcare to bring precision medicine closer to BMD and DMD patients. We have also studied novel lncRNA regulatory factors, shown their involvement in cell proliferation and differentiation and potentially muscle regeneration processes, and identified them as potential therapy biomarkers. Nevertheless, despite the increasing number of reported biomarker candidates, the FDA and EMA regulatory agencies require functional relevance of drug development. Thus, among lessons learned from clinical trials is the importance of natural history data to have a well-defined baseline and the awareness of current outcome measures limits. However, compared to 10 years ago, we have the privilege of several therapeutic compounds being available but with sometime limited effects or for eligible DMD patients only. Deep phenotyping and precision medicine will help in ushering along the next generation of treatments by identifying new therapies suitable to each individual patient based on a precise analysis of genetic, environmental and lifestyle factors.

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