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Efficiency of antisense-mediated exon skipping in normal and mutated DMD genes

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RIASSUNTO

Efficienza del processo di exon-skipping indotto da oligonucleotidi antisenso nel gene DMD, wild type e mutato

La distrofia mscolare di Duchenne:

La distrofia muscolare di Duchenne (DMD) è la più frequente malattia recessiva legata al sesso, con un'incidenza di circa 1 su 3500 maschi nati vivi.
I pazienti affetti da DMD sono solitamente costretti su sedia a rotelle dall'età di 12 anni e soccombono per insufficienza respiratoria o cardiaca verso la seconda decade d'età.
La distrofia muscolare di Becker (BMD) è la forma allelica della DMD con manifestazione più lieve; i pazienti possono arrivare a manifestare difficoltà motorie anche dopo i 40 anni, la qualità e l'aspettativa di vita sono decisamente migliori che non nella DMD (Wells et al., 2002).

Gene, proteine e struttura:

Mutazioni nel gene della distofina (*DMD*) portano alle due malattie. La proteina sintetizzata da questo gene ha un importante ruolo strutturale nell'ancorare il citoscheletro al sarcolemma favorendo l'integrità strutturale del muscolo durante la contrazione, è inoltre in parte responsabile della trasmissione della forza motrice prodotta al resto della massa muscolare e quindi nel risultante movimento.
Il gene *DMD* è situato sul cromosoma X, nella regione p21. È il gene più lungo dell'intero genoma umano, copre 2,4 megabasi e comprende 79 esoni intervallati da larghe sequenze introniche. Esistono almeno 7 forme alternative di prodotto trascitto, che comprendono 3 forme *full-length* (con risultanti proteine di 427 kDa) e almeno altri 4 promotori che codificano per le cosiddette "forme corte". I promotori delle forme *full-length* prendono il nome dal principale (ma non unico) sito di espressione; si hanno i promotori muscolare (M), cerebrale (C) e Purkinje (P). Le forme corte sono variamente espresse nei tessuti e coesistono spesso con le forme lunghe (Muntoni et al., 2003).
La proteina è strutturata in 4 domini distinti:

- Il domio N-terminale che lega l'actina nel citoplasma

- Il dominio spectrino-simile che consiste di 24 sequenze ripetute le quali conferiscono proprietà elastiche alla proteina
- Il dominio ricco in cisteine fondamentale per l'ancoraggio della proteina al sarcolemma
- Il dominio C-terminale o *coiled-coil* (CC) dove si hanno la gran parte delle interazioni con altre proteine.

La distrofina si lega ad un complesso reticolo di proteine situato sul sarcolemma; queste proteine di varia natura e funzione sono per la maggior parte trans-membrana e formano il cosiddetto "complesso associato alla distrofina" (DGC o DAPC). Le proteine del DGC legano sul versante citoplasmatico la parte terminale della distrofina, mentre sul lato esterno della cellula si legano a varie proteine della laminina basale (Roberts, 2001).

Filogenesi:

La distrofina è riconosciuta come la capostipite di una superfamiglia di proteine che è presente in tutto il regno animale; le regioni distintive di questa famiglia sono la regione ricca in cisteine e quella CC.
La filogenesi di questa superfamiglia origina da un antenato non metazoo da cui si sono separate due nuove famiglie proteiche: le distrofine e le distrobrevine.
Queste due famiglie hanno poi seguito strade diverse fra i vertebrati e gli invertebrati.
Nei vertebrati il ramo delle distrofine ha dato luogo prima di tutto alla sottofamiglia DRP2 (*dystrophin related protein 2*) e successivamente alla divisione fra utrofina e distrofina. Sul ramo delle distrobrevine invece si sono originati due geni paraloghi codificanti per l'α-distrobrevina e la β-distrobrevina.
Negli invertebrati il repertorio delle proteine è rimasto più semplice, mantenendo solo una famiglia per ramo, le proteine distrofino- e distrobrevino-simili. Tali proteine sono tuttavia molto conservate, ad indice della loro importanza funzionale e strutturale (Jin et al., 2007).

Animali modello:

Mutazioni nel gene *DMD* sono state riportate in molti animali vertebrati ed anche nei geni ortologhi degli invertebrati.
Il modello murino per lo studio della malattia è il topo *mdx*. Questo ceppo presenta una mutazione non senso nell'esone 23 che porta ad un messaggero troncato. Il facile allevamento gestione per le dimensioni e la gestione dell'animale lo hanno reso il modello più utilizzato nella ricerca per la DMD. Il

fenotipo risultante dalla mutazione tuttavia presenta molte differenze con la patologia umana.
Oltre al topo esiste anche un modello canino che riproduce più fedelmente la patologia umana e viene usato in studi più avanzati date le difficoltà di gestione di tali animali (Willmann et al., 2009).
Gli invertebrati hanno fornito negli anni importanti informazioni a livello molecolare e funzionale sulla distrofina e le proteine ad essa correlate (Wells et al., 2005).

Patogenesi:

La patogenesi della malattia vede l'insorgere di fibre necrotiche (solitamente in gruppo) e alcune piccole fibre centro nucleate (indice di rigenerazione muscolare). Con il passare del tempo il bilancio fra le capacità rigenerative del muscolo di far fronte alle fibre che vanno in necrosi viene meno e le fibre muscolari sono progressivamente sostituite da tessuto adiposo e fibroso (Bockhold et al., 1998).
Una delle principali cause di morte delle cellule si pensa sia associata alla conseguente fragilità della membrana delle cellule muscolari. L'influsso di calcio dall'ambiente extracellulare porterebbe ad un aumento della concentrazione citoplasmatica di questo ione, che con il tempo non potrebbe essere più controllata da meccanismi fisiologici e finirebbe con portare alla morte delle cellule (Deconinck et al., 2007).

Mutazioni:

Dal punto di vista genetico il gene *DMD* presenta un alto tasso di mutazioni (van Essen et al., 1992), le quali possono essere collegate alla malattia ricorrendo alla "teoria del registro di lettura" secondo la quale in presenza di una mutazione che fa slittare il normale registro di lettura del trascritto si viene a creare una proteina tronca e non funzionante che porta quindi al quadro patologico della malattia. Se la mutazione invece rispetta il registro di lettura allora si verrà a creare una proteina più corta ma in parte funzionante, come confermato da casi di pazienti con grandi regioni delete del gene con un fenotipo BMD o quasi non patologico, Questo è in parte dovuto al fatto che la proteina è costituita per più del 70% dalle sequenze ripetute del dominio spectrino-simile. È stato osservato che basta una minima porzione di tale regione della proteina per mantenere la sua funzionalità (Monaco et al., 1988).

Attuali terapie e ricerca:

Gli attuali approcci terapeutici per la DMD prevedono l'uso di farmaci come corticosteroidi per diminuire la manifestazione dei sintomi e migliorare il tono muscolare, ma questa categoria farmacologia non manca di controindicazioni.
Alcuni tipi di antibiotici sono in grado di far saltare il registro di lettura dove vi sia una mutazione non senso (come nel topo *mdx*) consentendo così di sintetizzare una proteina mutata ma parzialmente funzionale, ma nell'essere umano questa strada è ancora in fase di accertamento e la paura di effetti di *read-through* indesiderati fanno procedere con cautela (Yiu et al., 2008).
In assenza di distrofina si nota una aumentata espressione di utrofina, che potrebbe supplire alla mancanza della prima, ma non sono normalmente raggiunti i livelli richiesti per sopperire al fenotipo distrofico, alcune linee di ricerca si stanno incentrando su aumentare l'espressione di tale gene (Cossu et al., 2007).
Il trapianto di cellule miogeniche è stato ampiamente affrontato negli ultimi anni; le caratteristiche che tali cellule devono presentare sono: la capacità di ripristinare muscolo funzionante, non indurre risposta immunitaria, consentire la loro riproduzione per mantenere intatto un *pool* rigenerativo ed essere terapeuticamente valide come trattamento (Price et al., 2007).
Sono stati inoltre studiati diversi modi per indurre la produzione di forme funzionanti di distrofina per esempio tramite l'inserimento di geni esogeni tramite vettori virali, che andrebbero a fornire alle cellule distrofiche una copia da usare in sostituzione alla propria (Yiu et al., 2008).

Terapia tramite oligonucleotidi antisenso:
Negli ultimi anni un nuovo approccio si sta facendo strada, ovvero quello di indurre l'esclusione di ulteriori esoni dal pre-mRNA. A prima vista questo sembrerebbe insensato, ma se si pensa al fatto che così facendo si può ripristinare il registro di lettura originario, allora si può capire la validità terapeutica di questo approccio.
Gli oligonucleotidi antisenso (AOs) possono produrre effeti di "*exon-skipping*" durante il processamento del pre-mRNA. L'AO specifico si va a legare ad un sito appositamete studiato per interferire con il meccanismo di *splicing* della cellula, impedendo così l'inclusione di un esone nel trascritto finale, ottenendo così una proteina più corta ma parzialmente funzionale (Aartsma-Rus et al., 2007).
Questa sarebbe una terapia *ad hoc* da effettuare per ogni singola mutazione che si vorrebbe curare, ma i vantaggi terapeutici sono molti:

- Eliminerebbe il rischio delle terapie associate a trattamenti genici virali

- Permetterebbe il ripristino di tutte le forme di distrofina, non solo quella muscolare
- Manterrebbe la normale regolazione tessuto-specifica nella produzione di distrofina
- Seppure questa sia una terapia personalizzata la produzione dell'agente terapeutico non sarebbe dispendiosa e sarebbe molto semplice.

Non ultimo il fatto che già normalmente questo fenomeno si presenta in gruppi isolati di cellule nel muscolo distrofico, validandone le basi teoriche (Lu et al., 2000).
Verosimilmente i pazienti potrebbero essere portati verso un fenotipo più simile alla BMD se non mostrare segni di assenza della distrofia.
Per permettere la persistenza del trattamento gli AO sono stati modificati negli anni a livello della struttura molecolare dello scheletro o di modificazioni in 2', così da rendere più resistenti le molecole alla degradazione cellulare da parte delle endonucleasi presenti nelle cellule.

Scopo della tesi:

In questo lavoro di tesi si è andati ad indagare l'efficacia di alcuni oligonucleotidi antisenso progettati per escludere alcuni esoni da sequenze "sane". Le cellule dei pazienti utilizzate in questo lavoro invece presentano molte variazioni a livello di delezioni/mutazioni nel trascritto e si è voluto verificare l'applicabilità degli AO ad un contesto "mutato".
Le cellule dei pazienti sono state derivate da biopsie depositate nella banca di campioni bioptici di Telethon a Milano (in collaborazione con l'istituto Besta di Milano), mentre quelle dei donatori sani dalla banca Telethon di Padova (in collaborazione con la Dott.ssa M. Fanin).
Sono stati impiegati oligonucleotidi 2'-O-metil-fosforotioati modificati, sintetizzati in collaborazione con il laboratorio del Dott. S. J. Wilton.
Per fare questo si sono trasfettati dei mioblasti di pazienti in coltura per introdurre le molecole nelle cellule e consentire lo *skipping*.
Sono inoltre state effettuate prove di verifica della presenza di distrofina tramite immunofluorescenza.

Risultati:

Nelle prove effettuate si è andata a verificare l'efficienza dello *skipping* da parte degli oligo in cellule di mioblasti primari derivati da biopsie di donatori sani.
Successivamente gli stessi oligonucleotidi sono stati impiegati per trattare le cellule dei pazienti con la mutazione.
Le cellule sono state trasfettate con diverse quantità di AO, per saggiare eventuali differenze nei prodotti trascritti e sulla eventuale tossicità del trattamento.
Successivamente alla trasfezione sono state effettuate delle RT-PCR da cui si è andato a vedere la presenza o meno dello skipping.
Il disegno degli AO per le cellule sane non ha sempre riscontrato lo stesso effetto in cellule di paziente, anzi, tutt'altro. Si è visto che le mutazioni presenti nei pazienti portano spesso ad effetti diversi sui prodotti di *skipping* rispetto ai controlli sani, convalidando la nostra ipotesi che un disegno specifico su sequenze non mutate possa non essere terapeuticamente valido per le mutazioni di alcuni pazienti.

INTRODUCTION

Duchenne Muscular Dystrophy (DMD) is the most common X-linked recessive disease with an incidence of 1 in 3,500 male newborns. The pathogenesis includes progressive degeneration of skeletal muscle and muscular weakness. Regeneration of fibers becomes less frequent as the disease progresses and muscles are eventually replaced by adipose and connective tissues, accounting for the pseudohypertrophic muscles. Patients are usually confined to a wheelchair by the age of 11, and death from respiratory or cardiac failure is common in the late teens to early 20s (Wells et al., 2002).

The Becker Muscular Dystrophy (BMD, also known as Benign pseudohypertrophic Muscular Dystrophy) is the allelic form of DMD and has a milder clinical course and a slower disease progression than in Duchenne patients. BMD has been estimated to occur approximately one-tenth as frequently as DMD, with an incidence of 3-6 per 100,000 newborns. The majority of BMD patients initially experience difficulties between 5 and 15 years of age, although onset in the 3rd or 4th decade, or even later, may occur. By definition the affected patients remain ambulant until 16 years of age or later, thus allowing the clinical distinction from patients with DMD. Patients with BMD have a reduced life expectancy, but the majority of patients survive into at least the 4th or 5th decade.

Both types of dystrophies are the results of mutations in the *DMD* gene that codes for the dystrophin protein.

The DMD gene, protein structure and functions

Discovered in the mid-eighties (Monaco et al., 1986), the *DMD* gene is the longest known human gene, covering 2.4 megabases at locus Xp21; it represents 0.08% of the whole human genome and 1.5% of the X chromosome. The primary transcript measures about 2,400 kilobases and takes 16 hours to be transcribed (Tennyson et al., 1995). However, 99.4% of the gene for dystrophin is made of large introns and the coding sequence includes 79 exons that form a 14 kb mature mRNA. There are 7 known alternative transcripts, comprising three full-length ones that have the same number of exons but which are derived from three independent promoters in brain, muscle, and Purkinje cerebellar neurons (Figure 1 A). These three promoters consist of unique first exon spliced to a common set of 78 exons (Figure 1 B) and the names in each case refer to the major but not exclusive sites of expression; all three mRNAs code for a so-called full-length protein isoforms (427 kDa). In addition, dystrophin gene codes for 4

shorter isoforms, generated through alternative splicing events. These splice variants, which commonly occur in a tissue-specific way, are formed both through the exclusion of some exons (exon skipping) and by subversion of the reciprocal order of exons (exon scrambling (Muntoni et al., 2003).

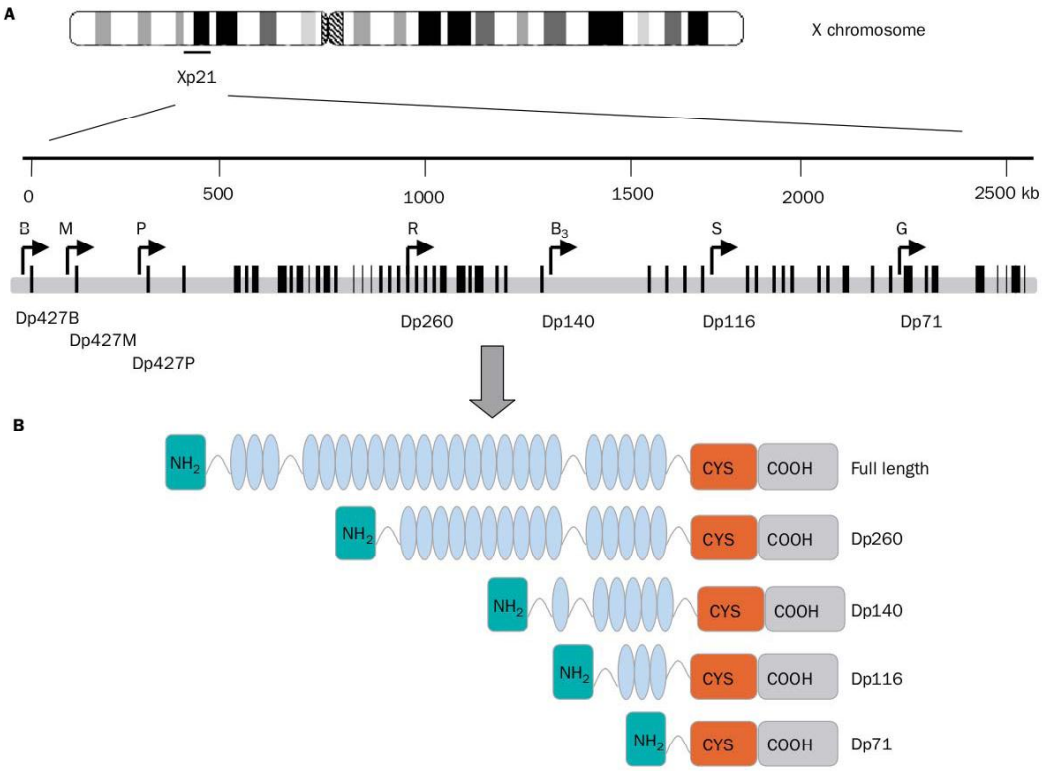

Figure 1 A: Genomic organisation of the dystrophin gene, located in Xp21. The black vertical lines represent the 79 exons of the dystrophin gene distributed over about 2.5 million bases. The arrows indicate the various promoters: in particular are brain (B), muscle (M), and Purkinje (P) promoters. B: The domain composition of the various dystrophin proteins is indicated. (Muntoni et al., 2003)

The full-length dystrophin is a member of the β-spectrin/α-actinin protein family, containing four distinct regions identified by sequence homologies and binding capabilities with several sarcolemmal/cytoskeletal proteins (see below) (Blake et al., 2002).

- Actin-binding domain: the amino-terminal 220 amino-acids of dystrophin, as well as utrophin and invertebrate dystrophins (see below) show clear homology to well known actin-binding regions of the spectrin and α-actinin families. The amino-terminal domains of dystrophin and utrophin have been shown by a variety of methods to bind filamentous actin with affinities below micromolar range and with marked preference for “non-muscle” forms of actin (Figure 3 B).
- Central rod domain: more than 70% of the protein length consists of a repeated motif similar to the triple helical repeats of spectrin, which is why this domain is also called “spectrin-like”. These α-helical coiled-coil repeats,

which are interrupted by four proline-rich hinge regions , are thought to give the molecule a flexible rod-like structure similar to β-spectrin.

- Cysteine-rich domain: the fourth proline-rich hinge is placed between the rod domain and a highly conserved constellation of motifs that constitutes a key feature for the dystrophin/dystrobrevin family. The region comprises the following: a WW domain named after its two conserved tryptophan residues (it usually binds proline-rich motifs), an EF hands (a helix-loop-helix structural domain found in a large family of calcium-binding proteins that consists of two alpha helices positioned roughly perpendicular to one another and linked by a short loop region that usually binds calcium ions) and a ZZ domain (a highly conserved and widespread zinc-binding motif first identified in dystrophin/utrophin and the transcriptional co-activator CBP) (Ponting et al., 1996). The structure of this region reveals a compact entity, with the WW domain and the EF hands intimately packed. Functional studies show that the cysteine-rich domain mediates the interaction between dystrophin and the intracellular tail of β-dystroglycan, the latter's PPPYVP motif binding to the WW domain; EF hand region increase the affinity of the WW domain cradling the site of interaction with β-dystroglycan (Rentschler et al., 1999). Recently it has been shown that ZZ domain is a second dystroglycan-binding site between dystrophin/utrophin and dystroglycan in addition to the WW domain mediated interaction with the extreme C-terminus of β-dystroglycan. This region is likely to be distinct from the well-characterized PPPYVP site due to its inability to compete for binding purified β-dystroglycan. This additional binding site further stabilizes the β-dystroglycan–WW domain interaction, which itself is supported by the EF hand region, and explains why the complete WW–EF–ZZ region appears to be required for full binding activity (Hnia et al., 2007). The generally accepted name is something of a misnomer, as only five of the fifteen cysteines (in human dystrophin) that give the region its name are highly conserved, four of these being metal ligands in the ZZ domain (Roberts, 2001).
- The carboxyl-terminal domain: the COOH terminus of dystrophin contains two polypeptide stretches that are predicted to form α-helical coiled coils similar to those in the rod domain. This domain has been named the coiled coil (CC) domain. Approximately 3-5% of proteins have coiled-coil regions. Coiled coils are well-characterized protein interaction domains. The CC region of dystrophin forms the binding site for dystrobrevin and may modulate the interaction between syntrophin and other dystrophin-associated proteins (Blake et al., 2002) (Figure 2). The first half of the C-terminal domain interacts

with the ZZ domain, strengthening its binding to the β-dystroglycan (Hnia et al., 2007).

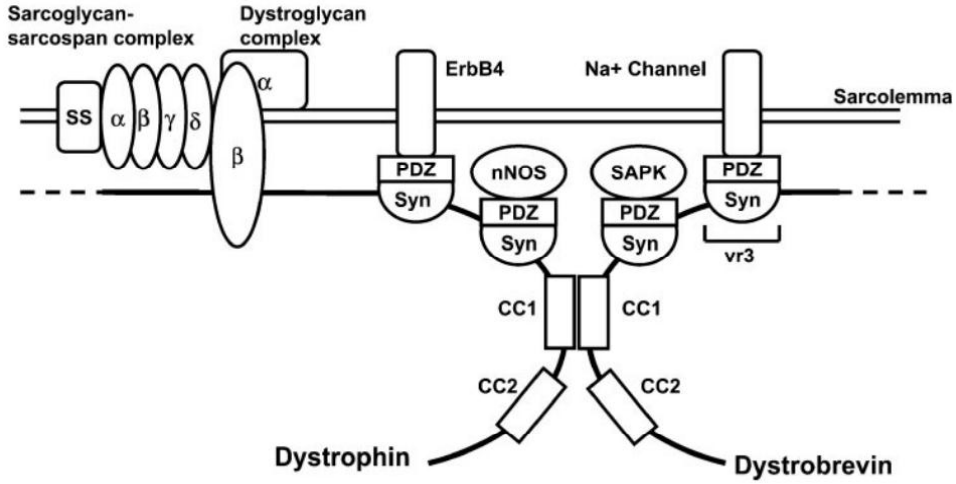

Figure 2 Protein interactions at the COOH terminus of dystrophin. (Blake et al., 2002)

All members of the dystrophin family appear to be membrane-associated. Vertebrate dystrophin is located at the cytoplasmic membrane of skeletal, cardiac and smooth muscle cells and at the central nervous system (CNS) in a subset of postsynaptic neurons (in cerebral cortex, hippocampus, and cerebellum). Shorter forms of dystrophin, generated by alternative promoter usage, are variously expressed: Dp260 is expressed in high concentrations in the retina, where it coexists with the full-length brain and muscle isoforms; Dp140 is expressed in brain, retina, and kidney tissues; Dp116 is only expressed in adult peripheral nerves at the outer surface of Schwann cells. Dp71 is detected in most non-muscle tissues including brain, retina, kidney, liver, and lung and is present in cardiac but not skeletal muscle (Muntoni et al., 2003).

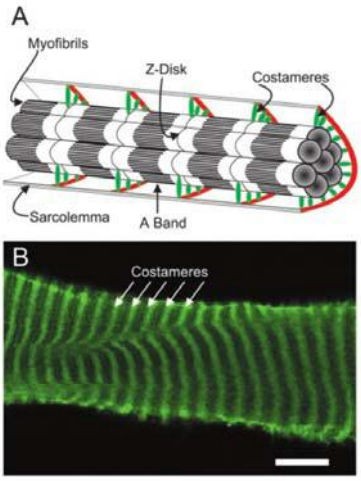

Figure 3 A: costameres structures coupling myofibrils to sarcolemma in periodic register with Z-disks.
B: sarcolemma stained with Ab to γ-actin to reveal the costameric cytoskeleton. (Ervasti, 2003)

Muscular dystrophin is part of a subsarcolemmal protein assemblies that circumferentially align in register with the Z-disk of peripheral myofibrils and physically couple force-generating sarcomeres with the sarcolemma in striated muscle cells. This lattice, called costamere (Figure 3 A), may function to laterally transmit contractile forces from sarcomeres across the sarcolemma to the extracellular matrix and ultimately to neighbouring muscle cells. Lateral transmission of contractile force would be useful for maintaining uniform sarcomeres length between adjacent actively

contracting and resting muscle cells comprising different motor units within a skeletal muscle. It is also logical that the sites of lateral force transmission across the sarcolemma would be mechanically fortified to minimize stress imposed on the relatively labile lipid bilayer. Other results have long suggested that costameres may coordinate an organized folding, or "festooning" of the sarcolemma, which again may minimize the stress experienced by the sarcolemmal bilayer during forceful muscle contraction or stretch (Ervasti, 2003). Shortly after identification of the DMD gene and dystrophin, it was shown that dystrophin could be tightly associated to at least 10 (trans)membrane proteins (Campbell et al., 1989). Since these proteins are co-localised with dystrophin at the sarcolemma, co-purified with dystrophin in stoichiometric amounts through several purification steps, and were diminished in biopsies from DMD patients and muscle of the murine dystrophin-deficient model (Ervasti et al., 1990), it was concluded that dystrophin functioned as part of a larger, hetero-oligomeric glycoprotein complex (Figure 4) that may serve to stabilize the sarcolemma against the repetitive stress imposed during muscle contraction.

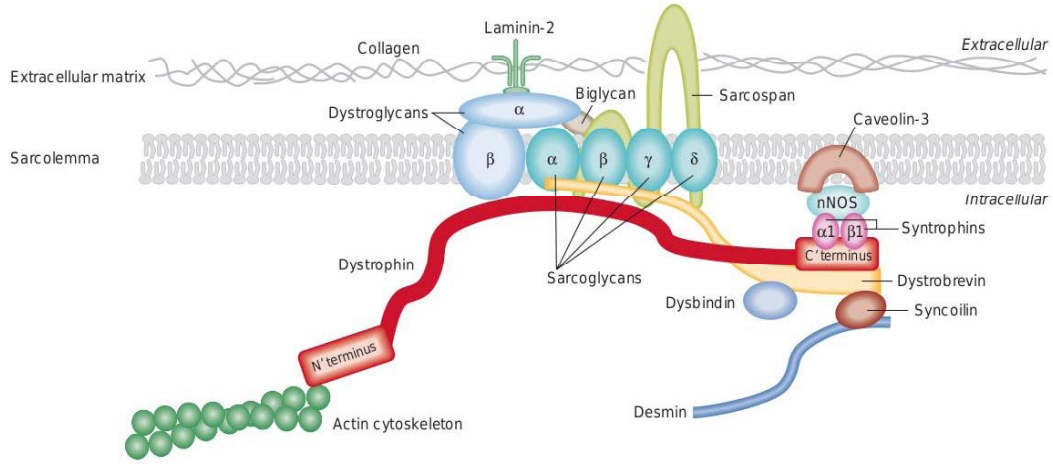

Figure 4 Dystrophyn interaction with DAPC at the sarcolemma membrane. (Nowak et al., 2004)

Dystrophin and its tightly associated proteins were collectively named the Dystrophin-associated Glycoprotein Complex (DGC or DAPC) (Campbell et al., 1989). A 156 kDa subunit presenting an extensive glycosylation, in addition with a 43kDa subunit, make a very tight association to dystrophin, which is why the subunits were renamed α- and β-dystroglycan, respectively. These make up the core of the DGC, establishing the transmembrane link between laminin-2 and dystrophin. Interestingly, their glycosylation patterns are developmentally regulated and largely correlate with the diversity of binding partners in different tissues (Winder, 2001). The characterization of the genes encoding the dystrobrevins and syntrophins (respectively the 88 kDa and 59 kDa dystrophin-

associated proteins) greatly benefited from comparative investigations into the molecular composition of the mammalian neuromuscular junction (NMJ) and electric organ of *Torpedo californica*, which preceded the discovery of dystrophin (Wagnera et al., 1993). Dystrobrevins and syntrophins are cytoplasmic proteins that bind directly to each other and to sequences within the carboxy-terminal domain of dystrophin. While syntrophins are thought to function as modular adaptors that anchor ion channels and signaling molecules to the DGC, no myopathy is associated with syntrophin ablation in mice (Kameya et al., 1999). In contrast, knockout of α-dystrobrevin results in a progressive myopathy, suggesting an important role in DGC function (Grady et al., 1999). Distinct but related genes encode the α-, β-, γ- and δ-sarcoglycan. The sarcoglycans are all single pass transmembrane proteins that co-assemble into a stable tetrameric complex. While its function is not fully understood, the sarcoglycan complex appears to strengthen interaction of β-dystroglycan with α-dystroglycan and dystrophin. Importantly, mutations in individual sarcoglycan genes lead to loss of the entire sarcoglycan complex (Ozawa et al., 2005). The 25 kDa dystrophin associated protein, named sarcospan, is also stably associated with the sarcoglycan complex. However, no human myopathy has been linked to mutations in the sarcospan gene and ablation of sarcospan in mice caused no muscle phenotype. Finally, α-dystrobrevin has been shown to directly interact with the sarcoglycan complex, which raises the possibility that the myopathy accompanying α-dystrobrevin ablation may arise from destabilization of an indirect linkage between dystrophin and the sarcoglycan complex (Ervasti, 2007).

Phylogenetics:

Dystrophin is now recognized as the founder member of a protein superfamily with representatives throughout the animal kingdom. The ~70-kDa signature structure of this family is the cysteine-rich domain followed by the coiled-coils region (Roberts, 2001) (Figure 5).

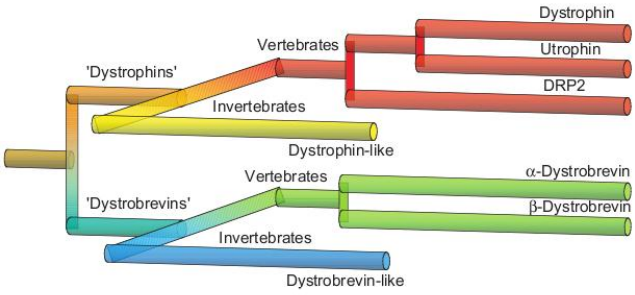

Figure 5 Phylogenetic tree of the dystrophin/dystrobrevin family, inferred from a tree constructed using sequences of the cysteine-rich and carboxy-terminal domains. (Roberts, 2001)

The superfamily includes various types of proteins structurally and/or functionally related to dystrophin:

- Utrophin: the utrophin gene (chromosome 6 in humans, 10 in mice) has the almost identical exon-intron organization than the *DMD* gene, the protein's structure is the same from NH to COOH terminus and several promoters with shorter isoforms have been described; utrophin and dystrophin are very likely paralogs that arose by duplication during vertebrate evolution. Utrophin expression is high during embryonic development but declines in adult skeletal muscle and is limited to the Neuromuscular Junctions (NMJ) and myotendinous junctions in normal adult skeletal myofibres; in early normal development, both dystrophin and utrophin are found at the sarcolemma of muscle fibres, but at birth utrophin is confined to the NMJ (Hirst et al., 2005). Some differences between utrophin and dystrophin actin binding have been identified: on one hand, utrophin lacks the additional actin-binding activity associated with the dystrophin rod domain; on the other hand, the NH terminus of utrophin contains a short extension not found in dystrophin which contributes to its affinity for actin (utrophin NH-terminal fragments thus bind cytoskeletal β-actin more strongly in vitro than dystrophin). The primary structures of the COOH termini of utrophin and dystrophin are also very similar, and this suggested that utrophin too might be able to bind members of the DGC. The similarities of structure and binding partners between dystrophin and utrophin have raised the possibility of some functional redundancy between the two proteins but clear functional roles for utrophin remain unclear (Blake et al., 2002).
- DRP2: the dystrophin related protein 2 (DRP2) is a further paralogue that retains only two of the spectrin repeats. Expression is largely confined to punctuate structures in the CNS and patches between the Cajal bands of Schwann cells in the Peripheral Nervous System (PNS). Its role in the CNS is unknown, but in the PNS DRP2 interacts with L-periaxin via its spectrin repeats (Jin et al., 2007).
- Dystrobrevin: dystrobrevins are a family of dystrophin-related and -associated proteins comprising alpha-dystrobrevin (α-DB) and beta-dystrobrevin (β-DB), both of which exist as multiple isoforms. There is also a recently discovered new gene, called γ-dystrobrevin, found to be distinct from α- and β-DB. In adult tissues, α-dystrobrevins have been characterized primarily as a component of the DGC in skeletal muscle cells. β-dystrobrevin is considered to be a non-muscular protein, which is abundant in brain, kidney, lung and liver. Dystrobrevins interact with proteins involved in signal transduction (e.g., α-dystrobrevins that bind neuronal nitric oxide synthase

(nNOS) and voltage-gated Na+ channels). The specific space-temporal expression profiles during mouse embryogenesis also suggest a developmental role for dystrobrevins. Both proteins lack the long N-terminal extension of dystrophins but contain homology with the cysteine-rich and carboxy-terminal domains of dystrophin and utrophin (Rees et al., 2007).

- Dystrotelin: in the past few years studies has shown the existence in most vertebrate genomes of exons encoding a novel, extremely divergent, possible member of the dystrophin/dystrobrevin family. This protein, called dystrotelin, shows significant similarity to dystrophin (30% identity, 49% similarity) and dystrobrevin (30% identity, 46% similarity) over the first 270 amino acids, corresponding roughly to the EF hands and ZZ domain. As there is 32% identity, 53% similarity between dystrophin and dystrobrevin over the same region, the three families of proteins appear to be equally related to each other. Further studies are required to better understand the function of dystrothelins and to resolve the trichotomy of dystrophin/dystrobrevin superfamily (Jin et al., 2007).

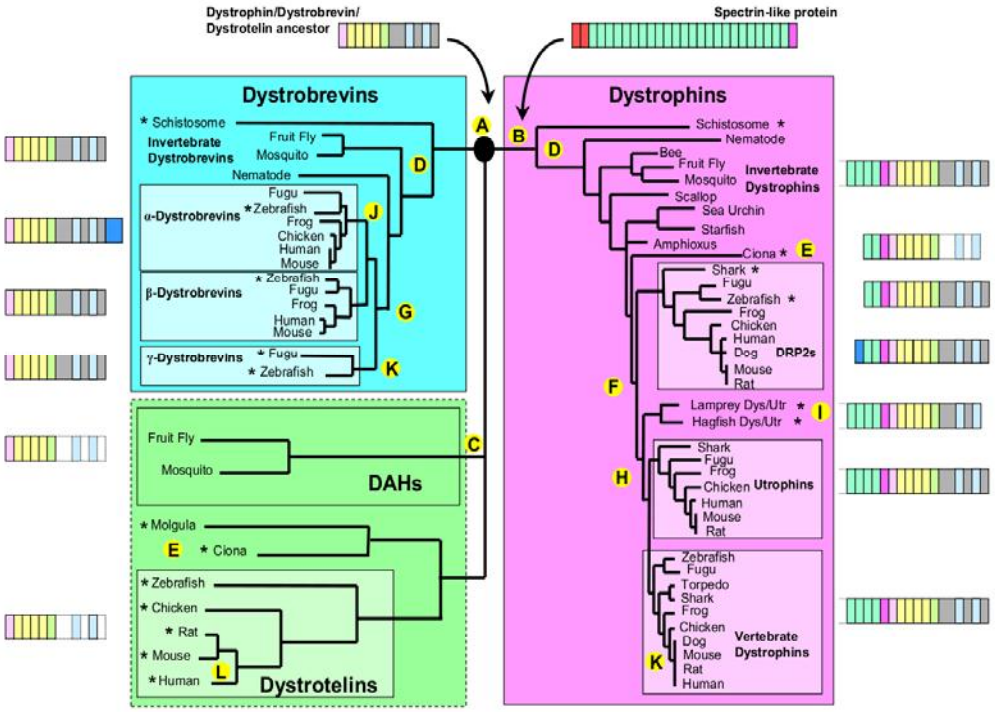

Figure 6 Phylogenetic tree with the unresolved point of divergence between dystrophins, dystrobrevins and dystrotelins. Phylogenetic distance is represented by the horizontal axis only. (Jin et al., 2007)

The evolution of the superfamily dystrophin/dystrobrevin (Figure 6) likely originated from a single ancestor (non-metazoan) with a gene containing all the signature structural elements still present in most contemporary representatives. Such ancestral protein probably functioned as a homodimer. Before the last metazoan ancestor, a duplication gave rise to the dystrophin and dystrobrevin

genes. This is the situation in most existing metazoans (including the cephalochordate amphioxus). In vertebrates a series of further duplications occurred; the first gave rise to DRP2 in the dystrophin branch and a second resulted in the separate dystrophin and utrophin genes (Roberts, 2001). On the dystrobrevins branch a duplication gave rise to α- and β-dystrobrevin's common ancestor, as well as to the γ-dystrobrevin recently discovered gene (Jin et al., 2007). Invertebrates, as it is the case with many gene families, have a simpler repertoire of proteins; all metazoans so far examined (including, for example, *Drosophila melanogaster* and *Caenorhabditis elegans*) have single dystrophin- and dystrobrevin-like molecules.

The possession of distinct dystrophin and utrophin molecules is a specifically gnathostome trait, arising from a duplication that occurred after our divergence from cyclostomes. The duplication which gave rise to the DRP2 gene occurred before this point in time, but after the divergence from amphioxus; thus the last common ancestor of cyclostomes and gnathostomes had two dystrophin-like molecules, namely DRP2 and a common ancestor of dystrophin and utrophin. Cyclostomes are likely to have subsequently lost their DRP2 gene.

Animal models:

Mutations on *DMD* gene have been reported in many vertebrates, as well as mutations in the hortologues genes of invertebrates, but only a few of them have been studied. Vertebrates (mammalian) are the main animal models currently used, although interesting features of invertebrates (see below) make them suitable for future approaches.

Mdx mouse:

The dystrophin deficient *mdx* mouse is the most widely used model of DMD; its small size and ease of husbandry make it a cost-effective model to investigate aspects of pathogenesis and treatment. The *mdx* mouse was first described by Bulfield and colleagues in 1984 when they noted mice with very high serum level of the muscle enzyme creatine kinase, indicative of muscle damage (this is also one of the main markers for DMD diagnosis). With the identification of the DMD gene and its protein product the *mdx* mouse was confirmed as having a dystrophin deficiency, which was subsequently shown to be due to a nonsense point mutation in exon 23. *Mdx* mice develops a severe myopathy at 2-3 weeks of age, which continues for approximately 5 to 6 weeks. After this peak the rate of degeneration slows down and then continues for the lifespan of the mouse, as shown by a continued raised level of serum creatine kinase. The majority of the limb musculature does not undergo major fibrosis and loss of function so that

the lifespan and general mobility of the *mdx* mouse is relatively normal, although there is a marked deterioration in older animals (Lefaucheur et al., 1995). This mild phenotype has lead to the *mdx* mouse being criticized as a poor model of DMD. There are many theories as to why the *mdx* mouse is less severely affected by the dystrophin deficiency compared to DMD patients, including differences in muscle regenerative capacity, increased utrophin expression, effects of size and/or the quadrupedal stance. However, one muscle, the diaphragm, is severely affected by the dystrophin deficiency showing dramatic fibrosis and fibres' loss. Other tonically active muscles, such as the soleus, also show marked fibrosis in the old *mdx* mouse (Wells et al., 2005).

A series of *mdx* variants (termed *mdx2Cv*, *mdx3Cv*, *mdx4Cv*, and *mdx5Cv*) were derived from mice treated with the chemical mutagen N-ethyl-nitrosourea. The main differences from the original *mdx* strain consist of larger variation in size, pseudomyotonia and some fibrosis detected in the hearts of *mdx2Cv* and *mdx3Cv* at the age of 4 weeks. The *mdx3Cv* strain also displays an abnormal breeding phenotype with reduced neonatal survival. The *mdx4cv* strain present lower frequency of revertant fibers (Willmann et al., 2009).

Canine models:

Spontaneous mutations of the dystrophin gene resulting in X-linked muscular dystrophy have been identified in several breeds of the domestic dog: the Golden Retriever (Cooper et al., 1988), the German short-haired pointer (Schatzberg et al., 1999) and the Beagle (Shimatsu et al., 2003).

The golden retriever muscular dystrophy (GRMD) is the best characterized dog model; the disease results from a single base pair change in the 3' consensus splice site of intron 6, leading to skipping of exon 7 and alteration of the reading frame in exon 8, which creates a premature stop codon (Sharp et al., 1992). Unlike the dystrophin-deficient *mdx* mouse, GRMD dogs suffer from a rapidly progressing fatal disease similar to DMD. There is, however, a large variation in disease severity as some pups survive only for a few days, while others are ambulant for months or even years. Incomplete muscle repair in GRMD results in progressive weakness and gait abnormalities at the age of 6-9 weeks, followed by muscle atrophy, fibrosis and severe contractures by 6 months. The dogs become less active than non-affected littermates at about 9 months of age. At this time, their gait is still uncoordinated and their limbs are abducted. Severely affected dogs have difficulties to rise and can walk only a few steps. At the age of 12 months, pharyngeal and oesophageal dysfunctions start to develop and respiratory capacity is decreased. Kyphosis develops by the 6^{th} month of age. Moreover, as seen in DMD patients, GRMD dogs display selective muscle

involvement although the pattern differs from that seen in humans. Myocardial involvement is much more evident in the golden retriever than in other animal models, matching very closely the cardiac complications often encountered in DMD patients (Moise et al., 1991).

Beagle dogs were mated with an affected golden retriever to obtain a breed of a smaller size, which is an advantage for some research purposes. One of these strains has been established in Japan (Canine X-linked Muscular Dystrophy in Japan $CXMD_J$). The survival rate of these beagle dogs is increased compared to golden retriever. In general, large breed dogs develop more severe clinical signs than small breed dogs. The diaphragm muscle is affected shortly after birth while in limb muscles abnormalities become evident only after 2 months of age. Cardiac involvement in the $CXMD_J$ dogs is milder and has a slower progression than that described for GRMD dogs (Shimatsu et al., 2005).

Feline models for DMD:

The dystrophic cat, which lacks approximately 200 kb of the dystrophin gene, is affected by ‘‘Hypertrophic Feline Muscular Dystrophy” (HFMD) (Winand et al., 1994). In HFMD the levels of creatine kinase in the blood increase by the age of 4-5 weeks, whereas muscle involvement becomes apparent only at the age of 10-14 weeks. The name of the disorder reflects the development of extensive muscle hypertrophy and affected animals eventually die due to compression of the oesophagus by the hypertrophied diaphragm or because of impaired water intake caused by glossal hypertrophy. Histological analysis has revealed the presence of local foci of necrosis and regeneration but no signs of fibrosis were seen. Although cardiac failure is rare, hearts are hypertrophic in 9-month-old cats. Thus, the pathology of HFMD cats does not resemble DMD as closely as the GRMD dog because it lacks the hallmarks of generalized muscle wasting (Willmann et al., 2009).

Other vertebrate models of DMD:

The relative ease and reduced cost of generating genetic mutants in the zebrafish (*Danio rerio*) compared to the mouse, as well as the rapid muscle development at a time when the embryo is effectively transparent, has led to this species being investigated as an animal model of DMD (Wells et al., 2005). Zebrafish are attractive as models of myopathy because they have high skeletal muscle content and express orthologues of most human DGC proteins with similar membrane localization. While gene-targeting technologies are not currently available, equivalent experiments can be carried out using oligonucleotide analogues (morpholinos) that disrupt the translation of specific mRNA transcripts. This technique has been used to create dystrophin-deficient

zebrafish that have an unstable DGC, bent morphology and lower activity (Guyon et al., 2003).

Invertebrate models:

The two invertebrate "workhorses" widely used in genetics research, *Drosophila melanogaster* and *Caenorhabditis elegans*, have also been used as animal models of DMD. Most of the work with the invertebrates has been devoted to identifying the homologous genes for dystrophin and DAG and understanding their role using various mutants. Conservation of sequence and gene structure shows the fundamental importance of many of these genes in maintaining muscle function. The muscles of *C. elegans* have a sarcomeric structure and similar proteins to mammalian striated muscle, despite their being formed by single mononucleated cells. Mutation of the dystrophin-like gene (*dys-1*) leads to a progressive locomotor defect due to widespread destruction of the muscles of the body wall. *C. elegans* has been used to examine genes that can modify the effects of the dystrophin deficiency and has recently been used to screen a range of compounds for their ability to reduce muscle degeneration. This latter showed that although none of the drugs tested reduced the locomotor impairment, the corticosteroid prednisone (which is already in DMD support treatment) reduced the number of abnormal cells, thus confirming the potential utility of this model. As the authors noted, the fact that *C. elegans* has no vertebrate-like immune system and does not have an inflammatory response, strongly suggests that a beneficial effect of prednisone could also be exerted directly on the muscles (Wells et al., 2005).

Mutations and pathogenesis in DMB/BMD:

Patients with DMD have very little or no detectable dystrophin whereas BMD patients have dystrophin of altered size and/or in diminished quantity. However, etiopathogenesis may be more complex than a simple loss of dystrophin. Studies have shown that several of the glycoproteins that interact with dystrophin are also absent in these disorders (Ervasti et al., 1990) and they have been suggested to be directly involved with increased calcium influx in the dystrophic fibers. Thus, the loss of dystrophin may be just the first of many steps that ultimately lead to muscular dystrophy.

Approximately 60 to 65% of the mutations that cause DMD/BMD are large deletions in the dystrophin gene. Their distribution along the gene show that they are no randomly distributed and occur primarily in two "hot spots": one in the rod domain (~80%) and one near the 5' end (~20%) of the gene. The 200-kb region covering intron 44, exon 45, and intron 45 is the major deletion

breakpoint region of the gene. Most of the larger deletions initiate at the 5' end of the gene. Initial studies showed no immediate correlation between the size/location of the deletion and the severity of the phenotype. Furthermore, sequences deleted in DMD patients overlapped with deletions in BMD patients (Figure 7 A). The existence of the two allelic forms was then explained by the so-called "reading frame hypothesis" (Monaco et al., 1988), i.e., it was proposed that if a deletion disrupts the translational reading frame of the dystrophin mRNA then a C-terminally truncated, non-functional protein will be synthesized, resulting in the more severe DMD. On the other hand, in BMD patients the deletion maintains the translational reading frame and thus a semi-functional protein is produced. The hypothesis explains the phenotypic differences observed in ~90% of the DMD/BMD cases, including one of the largest rod-domain deletions identified (35 exons) in a mild BMD patient. Exceptions to the reading frame hypothesis (~8-9%), involving an out-of-frame exon deletion with a BMD phenotype (for example exon 3 to 7 deletions) (Malhotra et al., 1988), have been explained with an alternate splicing mechanism or a new cryptic translational start site. A small number of DMD patients with in-frame deletions have also been identified. The more severe phenotype in these patients may be due to the overall effect of the deletion on the protein conformation or be the result of message instability.

Some phenotypic variability occurs in patients who share identical gene deletions. The out-of frame deletion of exon 45, one of the most commonly observed DMD deletions, has also been associated with BMD phenotypes (Prior et al., 1997). Some genetic variability might be due to modifier genes that affect splicing or other molecules involved in destruction of damaged muscle fibers, muscular regeneration, or in the cellular response to different hormones.

The DMD gene has an unusually high mutation rate, between 10^{-4} and 10^{-5} (van Essen et al., 1992). This could be due to the large gene size, particularly the very large introns, but other factors are also likely involved. For example, the observed non-random deletion pattern may reflect domain-associated variation in chromosomal stability (Prior et al., 2005); besides, complications related to the maintenance of replication, correct transcription and proper splicing of such a large gene might play an important role. Partial gene duplications have been revealed in ~5 to 10% of patients (Hu et al., 1990). Unlike the deletion distribution, duplications occur more frequently at the 5' end of the gene than in the central region (Figure 7 B).

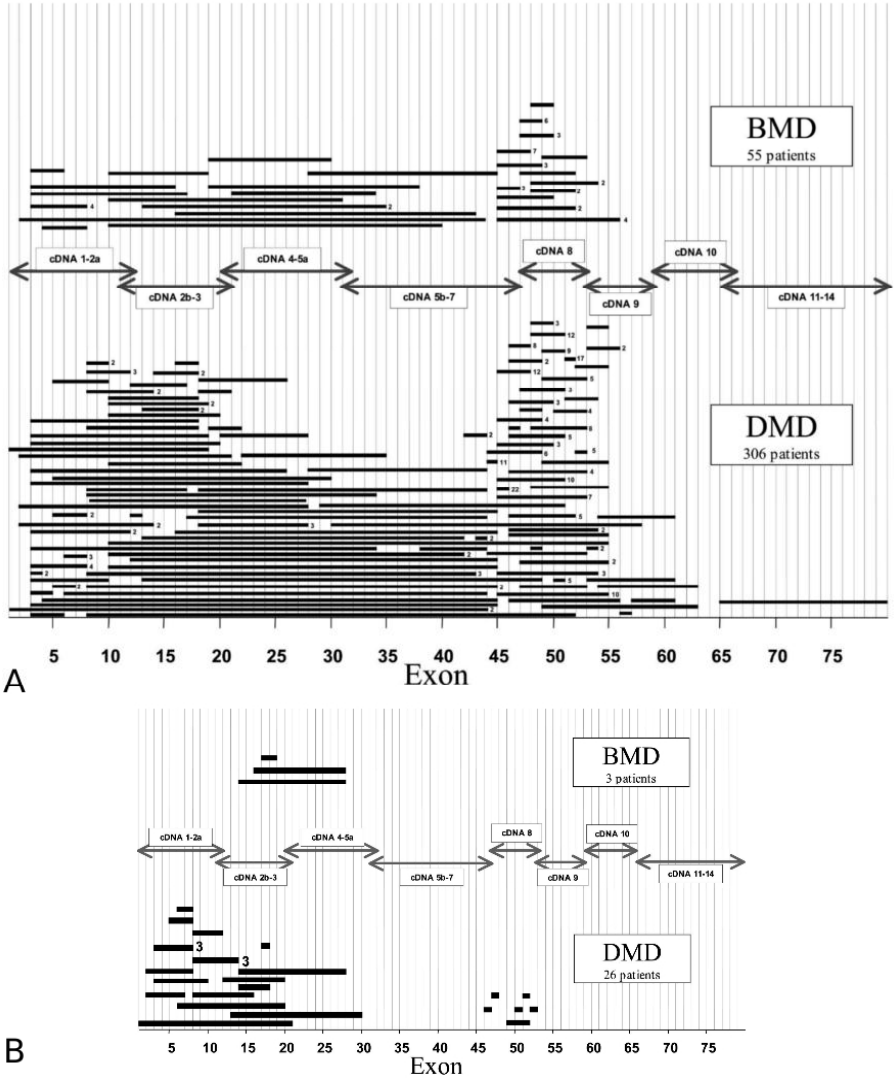

Figure 7 Distribution of deletions (A) and duplications (B) in the dystrophin gene in DMD and BMD patients. Each bar represents a mutation observed in a patient. The number to the right of the bar indicates the number of independent patients sharing mutation of the same exon. (Prior et al., 2005)

The deletion and duplication distribution has been demonstrated in different populations and ethnic groups. Out-of-frame duplications in DMD patients and in-frame duplications in BMD patients have been observed, suggesting that the reading frame hypothesis also holds true for duplications. As expected, there are also reports of small mutations – point mutations, small deletions and duplications – found in DMD and BMD patients without large deletions or duplications (Prior et al., 1995). Missense mutations are rare in the dystrophin gene, even amongst BMD patients. Several base changes causing significant amino acid substitutions have been reported in the dystrophin gene, but the majority of these resulted to be polymorphic changes. DMD missense mutations described in exons 3 and 16 have supported the important role of an intact actin-binding domain and of a little portion of the rod domain for dystrophin function (Prior et al., 2005) (Wang et al., 2000).

Despite the almost twenty years passed from the isolation of DMD gene, comprehensive understanding of the mechanism leading from the absence of dystrophin to the muscular degeneration is still lacking. In patients affected by DMD, muscle biopsy characteristically demonstrates necrotic or degenerating muscle fibers, often observed in clusters. These necrotic fibers are surrounded by macrophages and CD4+ lymphocytes. Small immature centrally nucleated fibers are also observed, reflecting the ongoing muscle regeneration that in the early phases of the disease leads to a balance between necrotic and regenerative processes. As time goes by, though, the regenerative capacity of the muscles becomes exhausted and muscle fibers are gradually replaced by connective and adipose tissue (Bockhold et al., 1998). The manifestations of Duchenne muscular dystrophy are hence considered to be the result of an imbalance between muscle fiber necrosis and myoblast regeneration.

Early descriptions of muscle histopathology (with focal lesions in the plasma membrane overlying wedge-shaped defects in muscle fibers known as "delta lesions") and raised levels of muscle enzymes in Duchenne muscular dystrophy patients have long been interpreted as reflecting excessive fragility of the muscle fibers. The discovery of dystrophin and other members of the dystrophin-associated glycoprotein complex scaffolding supported the view that the absence of one of these proteins could compromise the muscle membrane integrity of the fibers, particularly after sustained contractions.

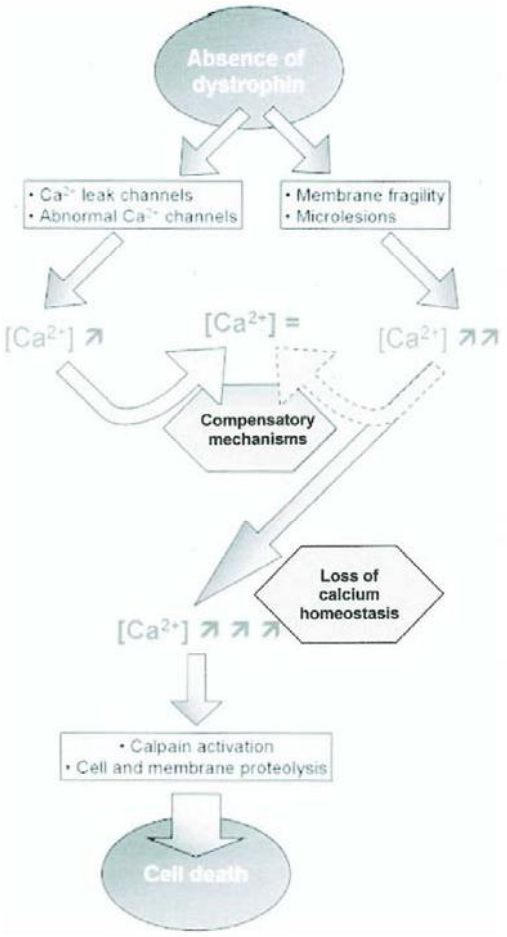

Figure 8 Proposed pathophysiology of dystrophinopathies. (Deconinck et al., 2007)

Documentation of calcium accumulation and of hypercontracted fibers in muscle biopsies of Duchenne muscular dystrophy patients has led to the investigation of the possible role of calcium in Duchenne muscular dystrophy. Increased influx through a dystrophin-deficient membrane has been demonstrated (Franco et al., 1990). This influx seems to occur mostly through mechanosensitive, voltage-

independent calcium channel. However, despite increased influx, low to normal calcium concentration can be maintained within the fiber cytosol, reflecting the robustness of the calcium homeostatic mechanisms. Still, if mechanical stress induces microlesions in the fiber membrane high influx of extracellular calcium inevitably occurs, overriding the capacity to maintain physiologic cytosolic Ca^{2+} concentration. Sustained increase in cytosolic calcium concentration leads to activation of proteases, particularly calpains, resulting in the destruction of membrane constituents that, in turn, will increase calcium entry. Excessive calcium may then lead to cell death (Figure 8). In line with the calcium hypothesis, several treatment trials with different calcium-blockers (e.g., diltiazem) have been tested in Duchenne muscular dystrophy, but have demonstrated almost no clinical benefit. On the other hand, overexpression of calpastatin (endogenous inhibitor of calpains) has been demonstrated to reduce necrosis in *mdx* muscle (Deconinck et al., 2007).

Revertant fibers:

An interesting phenomenon that occurs in ∼40% of DMD patients (Fanin et al., 1995) is the presence of "revertant fibers". These are defined as centronucleated fibers (deriving from regeneration processes) that express dystrophin (Hoffman et al., 1990). They represent only a little percentage of all muscle fibers and this phenomenon is not limited to humans, but has also been observed in the *mdx* mouse and the CXMD dog (Wilton et al., 1997). Immunofluorescent analysis using a wide group of antibodies specific to the N- and C-terminal regions of dystrophin showed that these domains are expressed in revertant fibers (Thanh et al., 1995). However, antibodies recognizing portions of dystrophin encoded by exons near the *mdx* mutation (nonsense mutation in exon 23) typically fail to detect the revertant dystrophin. These studies therefore suggested that revertant fibers express dystrophins that arise from alternatively spliced (in-frame) transcripts lacking both the mutant exon and a variable number of adjacent exons (Lu et al., 2000).

Therapeutic approaches for DMD:

Pharmacological therapies:

Corticosteroids :

Prednisolone, prednisone and deflazacort are the only drugs shown to be able to delay the progression of DMD, while easing its symptoms. The specific mechanisms by which corticosteroids improve strength in affected patients are not known, but various possibilities have been proposed. These include alteration of gene regulation in muscle fibers, slowing down of skeletal muscle

breakdown, decrease in the number of cytotoxic T cells, lowering of cytosolic calcium concentrations and increase myogenic repair. Non-randomized studies of long-term daily corticosteroids suggest that ambulation may be prolonged by up to three to five years and that life expectancy is improved. Corticosteroids also appear to have a positive effect on the complications associated with DMD.

Proteasome inhibitors:

Bonuccelli and coworkers (Bonuccelli et al., 2003) explored the use of proteasome inhibitors as a therapy for DMD on the premise that, in the absence of dystrophin, members of the DGC are degraded through an unknown pathway that leads to their reduction in dystrophic muscle. They initially investigated the continuous systemic treatment with the proteasome inhibitor MG-132 in *mdx* mice; other proteasome inhibitors have then been explored, leading to decreased damage of the muscle membrane and improved muscle integrity. These findings corroborate the proposal that protein degradation in dystrophin-deficient muscle is mediated by the proteasomal pathway and open up a new avenue for possible therapeutic approaches.

Aminoglycoside antibiotics:

Between 5% and 15% of DMD cases are caused by premature stop codons, and so the use of aminoglycoside antibiotics (for example, gentamycin and negamycin), which promote translational readthrough of stop codons, has been investigated. Despite hopeful results in *mdx* mice – 6% dystrophin-positive fibers (Arakawa et al., 2003); 10–20% of normal dystrophin levels (Barton-Davis et al., 1999) – no dystrophin expression has been achieved in human studies of DMD and BMD patients and a replication of the *mdx* results have not been forthcoming (Dunant et al., 2003).

PTC124 is a new orally administered drug that promotes ribosomal read-through of stop codons and full-length dystrophin expression and decreased CK levels with PTC124 has been successfully detected in phase 2 trials.

The main concern regarding the use of read-through stop codon drugs is of course the theoretic risk of a global read-through of physiological stop codons (Yiu et al., 2008).

Utrophin overexpression:

In the absence of dystrophin, utrophin is upregulated, but not to a level sufficient to compensate functionally for the loss of dystrophin and prevent the progression of muscular dystrophy. Increasing the expression of utrophin in the muscles of *mdx* mice by three- to four-fold is sufficient to prevent or dramatically reduce muscular dystrophy pathology (Cossu et al., 2007).

Various factors that increase utrophin expression are being explored, such as heregulin and L-arginine, but most are still in the very early stages (Yiu et al., 2008).

Transplant of myogenic cells:

Regeneration in skeletal muscle is driven by its resident population of myogenic stem cells, termed "satellite cells", located between the basal lamina and sarcolemma of mature skeletal muscle fibers. These are considered multipotent stem cells, as they not only can enter the myogenic differentiation pathway but can also be induced to enter adipogenesis and osteogenesis. Normally quiescent, upon activation satellite cells rapidly proliferate and originate large number of myogenic precursor cells, named myoblasts. These latter can also be cultured and expanded in vitro for several passages.

In the recent past several different stem cell populations, derived from muscle but also from other compartments, have been assayed for their ability to treat muscular dystrophy. In order to correct the dystrophic phenotype, transplanted cells must fuse to existing, or form new, myotubes. Upon fusion, the contribution of genetically normal myonuclei to the muscle myofiber should result in the production of a functional dystrophin protein. At the same time, however, transplanted cells should be able to replenish the satellite cell pool of the diseased muscles.

Past experiments involving myoblast transfer to treat DMD (Law et al., 1990) failed to show substantial physiological correction of the dystrophic phenotype.

More recently, different groups have demonstrated that the transplant of a small number of freshly isolated (i.e., non expanded in vitro) satellite cells can greatly contribute to muscle regeneration in *mdx* mice (Collins et al., 2005) (Sacco et al., 2008).

Other studies have been focused on the use of different stem cells (Figure 9): muscle side population (SP) cells (Gussoni et al., 1999), bone marrow derived cells (Ferrari et al., 1998), multipotent mesenchymal stem cells (MSCs) (Caplan, 1991), muscle derived stem cells (MDSCs) (Torrente et al., 2001), vessel associated stem cells (mesangioblasts) (Sampaolesi et al., 2006), embryonic stem (ES) (Bhagavati et al., 2005).

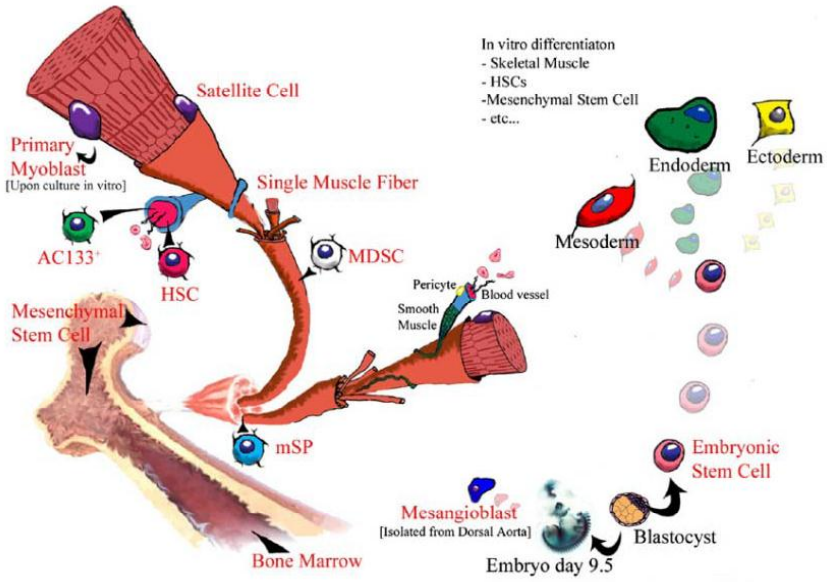

Figure 9 Diversity of myogenic stem cell populations. (Price et al., 2007)

At present, however, several problems remain to be solved before envisaging a cell-based trial in humans.

Gene therapy:

Gene addition:

The use of recombinant viral vectors that carry critical regions of the DMD gene has being explored since mid 1990s. As only a small transcript size can be incorporated into most available vectors, microdystrophin and minidystrophin genes have been created, leading to restoration of dystrophin expression in murine and canine models. The main challenges in this form of therapy include the likely need for immunosuppression and the optimization of recombinant vector delivery to multiple muscle groups. The use of non-viral vectors has also been described (Yiu et al., 2008).

Exon skipping by means of antisense oligonucleotides:

Antisense oligonucleotides (AOs) can sterically inhibit gene expression by hybridizing to target RNA sequences at key sites for the pre-mRNA maturation or for the translation process (Aartsma-Rus et al., 2007).

Researchers have hence tried to use AOs to redirect dystrophin splicing and exclude one or more exon(s) (exon skipping) in order to restore the reading frame of the mRNA and produce a slightly shorter but partially functional protein. In other terms, this approach would try to convert DMD patients into their milder counterpart, BMD (Figure 10).

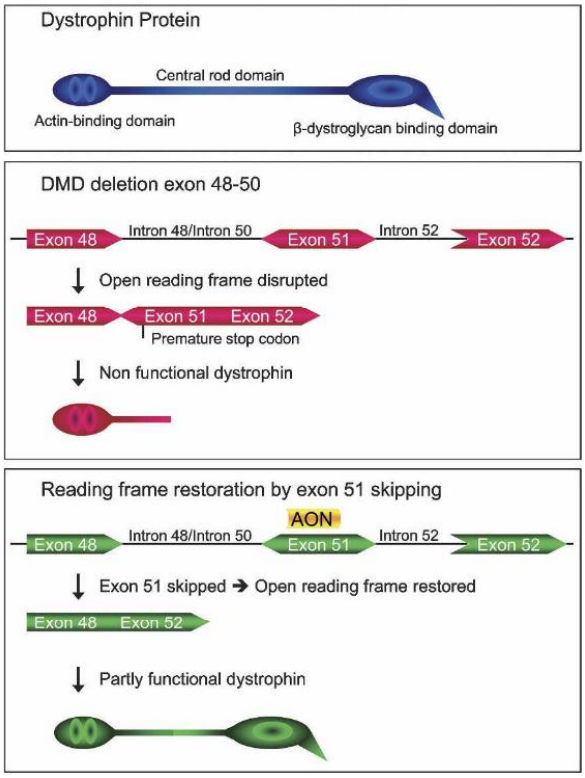

Figure 10 Restoration of the reading frame using antisense oligonucleotides (AON). (Aartsma-Rus et al., 2007)

Normal occurring alternative splicing of dystrophin pre-mRNA in revertant fibers has had a great influence in exon skipping studies, suggesting the strategy of restoring the reading frame.

AOs used in exon skipping target pre-mRNA sequences involved in the splicing process, preventing the inclusion of one or more specific exons. Acceptor, donor and branch sites should be the obvious target for exon skipping (Figure 11 a), but Exonic Splicing Enhancer (ESE) are also common targets (Figure 11 c).

AOs for exon skipping are relatively short (13–25 nucleotides) and hybridize (at least in theory) to a unique sequence in the total pool of targets present in cells. Although it is not a complicated matter to synthesize phosphodiester oligonucleotides, their use is limited as they are rapidly degraded by the intracellular endonucleases and exonucleases, usually via 3'→5' activity. In addition, the degradation products of phosphodiester oligonucleotides, dNMP mononucleotides, may be cytotoxic and also exert antiproliferative effects (Dias et al., 2002). For this reason, modified AOs have been widely studied.

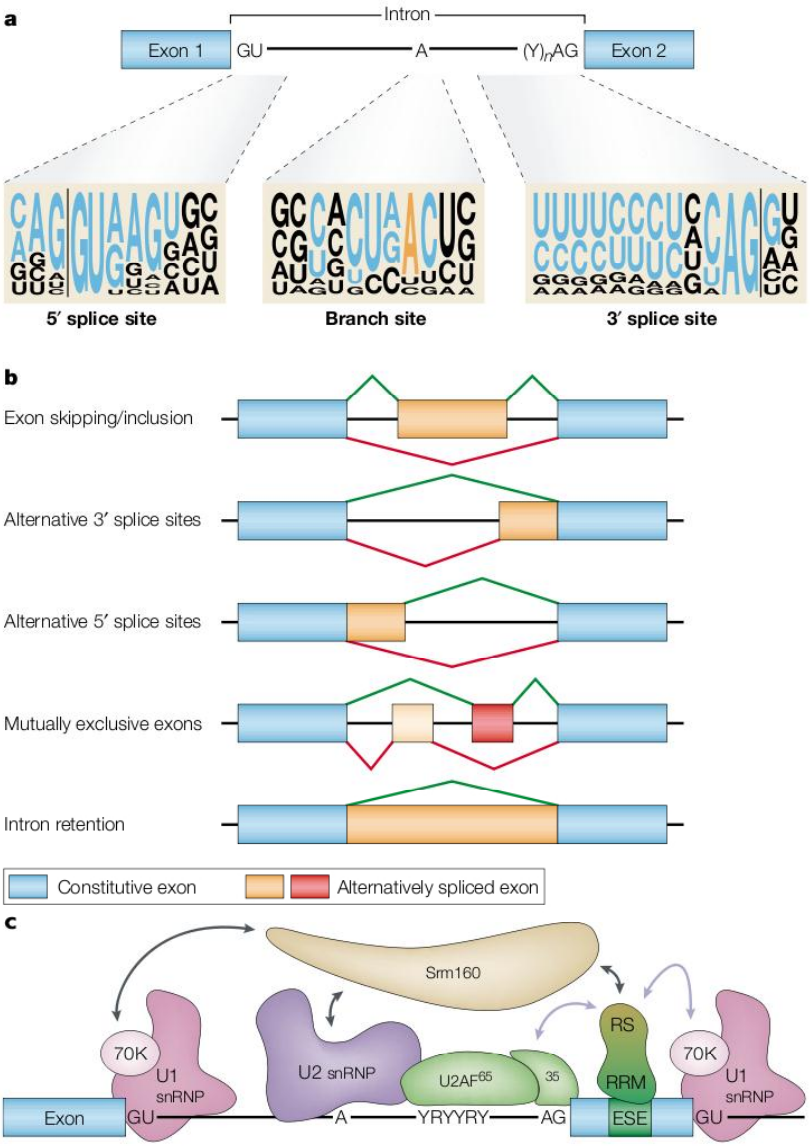

Figure 11 a: Conserved motifs at acceptor (5'), donor (3') and branch site. b: common modes of alternative splicing. c: protein interaction of exonic splicing enhancer (ESE). (Cartegni et al., 2002)

First-generation phosphorothioate modifications used a substitution of a non-bridging phosphoryl oxygen of DNA with sulphur, increasing resistance to nuclease digestion and prolonging half-life (Figure 12 A). Second-generation AOs have then been designed, showing improved stability and efficacy thanks to chemically modification of the phosphodiester-linkage, the heterocycle or the sugar (Figure 12 B). So far, modifications made to the ribose, and in particular, the 2'-position, seem to be the most important in improving the pharmacokinetic characteristics of second-generation AOs. Third generation chemical modifications such as PNAs (Peptide Nucleic Acids) and PMOs (Phosphorodiamidate Morpholino Oligos) (Figure 12 C) promise further improvements (Gleave et al., 2005).

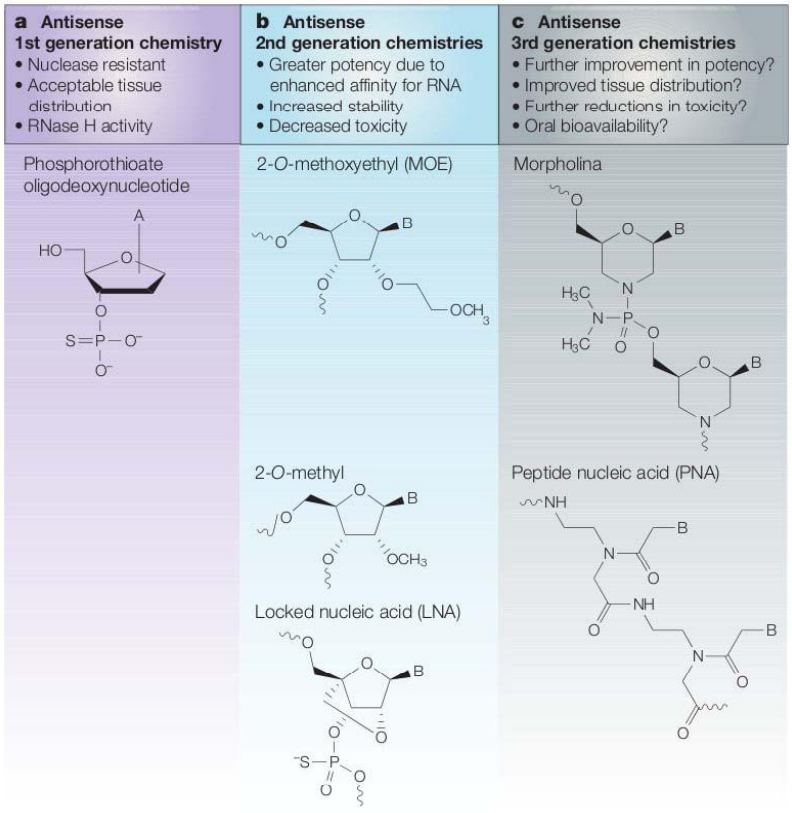

Figure 12 First, second and third generation AO. (Gleave et al., 2005)

Successful skipping has been demonstrated in cultured *mdx* myotube (Wilton et al., 1999), the *mdx* mouse (Lu et al., 2003), and cultured muscle cells derived from DMD patients (Aartsma-Rus et al., 2003). Recent investigations into double-exon and multi-exon skipping (skipping of numerous successive exons) have enhanced the technique to treat a greater number of dystrophin mutations (Aartsma-Rus et al., 2004).

van Ommen and colleagues recently proceeded with the first human trial with successful results, by injecting a 2'-O-methyl–modified ribose molecule with a full-length phosphorothioate backbone (2OMePS) into the *tibialis anterior* muscle (van Deutekom et al., 2007), obtaining 17 to 35% of dystrophin expression. Muntoni and colleagues recently finished a second trial using a PMO (Kinali et al., 2009) with dystrophin restoration up to 42%.

One of the major concern about AOs was that no dystrophin rescue could be observed in the heart muscle, but last year Lu and colleagues demonstrated *in vivo* a "nearly total" restoration of functional dystrophin (even in cardiac muscle) using a peptide-tagged phosphorodiamidate morpholino oligomer (PPMO) in *mdx* mice (Wu et al., 2008).

AIM OF THE WORK

Up to now virtually all AO designing procedures for exon skipping in the human DMD gene have been tested on normal human cells. There have been recent indications that the different genomic re-arrangements (deletion, duplications, point mutations, *etc.*) present in patients could affect the efficiency of the desired skipping processes, but this aspect has been barely studied so far. In this work we hence compared the results obtainable in wild type controls and in myoblasts from patients carrying different types of mutations, when using AOs designed from the wild type DMD sequence with the currently available software tools.

MATERIALS AND METHODS

Cell cultures:

Unless otherwise specified, all cell culture reagents were from Invitrogen™.
Medium used for cells proliferation was called “aneural medium” (see below for composition).
Biopsies from healthy donors were used to obtain wild type cells (called MCQ), these cells were used to test the AOs in a non mutated context. Muscle specimens were obtained from the Telethon biopsy repository of Padua (collaboration with Dr. Marina Fanin). Patients’ cells (Table 1), derived with the same procedure, were then sent us as frozen cultures by the Telethon Cell Bank in Besta Institute in Milan.
Frozen biopsies were rapidly thawed in a water bath at 37°C, residual dimethylsulphoxide (DMSO) was washed away rinsing biopsies in rich medium (70% M199 and 30% FBS) and muscle specimens were incubated in a freshly prepared mixture of 80% rich medium plus 20% human plasma (this medium was filtered through a dish with 0.22 µm pore directly over the specimens) into a well of a 6 well plate. Several days after plasma clot formation, myoblast cell proliferation was observed, muscle specimens were then trimmed into pieces of about 1 mm^3 and placed on a new well. Growing cells were fed with aneural medium, replaced three times a week. Tissue fragments were repositioned every 3-4 days and cells were trypsinized to allow them to proliferate without differentiate.
Aneural medium was composed of:

- 60 % Dulbecco's Modified Eagle Medium (DMEM)
- 20% M199
- 20% Fetal Bovine Serum (FBS)
- Insulin 10 ng/ml final
- bFGF 2 ng/ml final (from PeproTech ©)
- EGF 10 µg/ml final (from PeproTech ©)

FGFs (Fibroblast Growth Factors) are known to be potent regulators of myogenic cell proliferation and differentiation. When used in primary muscle culture they have been shown to delay, but not inhibit, myoblasts fusion; bFGF in human muscle appears to exerts both proliferative and trophic influence (Sheehan et al., 1999).
Insulin is a factor for growth and maturation of many types of cells; the addition of supra-physiologic doses of insulin promotes differentiation of muscle cells of various animal species.

EGF (Epidermal Growth Factor) alone was reported to be the most important factor influencing muscle differentiation in human satellite cells (Ham et al., 1990).

Interestingly the presence of those three factors can increase the total number of cells while a combination of only two of them influence only morphology (Askanas et al., 1985).

All media were added with a mixture of antibiotics called PSFG containing:

- Penicillin 10 U/ml final
- Streptomycin 0,1 mg/ml final
- Fungizone 0,8 µg/ml final
- Gentamycin 0,15 mg/ml final

Frozen cells were rapidly thawed in a water bath at 37 °C and diluted in approximately 5 ml of DMEM before being pelleted with a 10 minutes spin at 400×g. Cells were resuspended and plated in aneural medium 80% plus 20% of macrophage-conditioned medium (MCM). This latter is known contains muscle-specific growth factor that increase primary myoblast proliferation and enhance their myogenic differentiation (Cantini et al., 2002). Previous work has also demonstrated that MCM could be used in cultures of primary myoblasts from DMD patients to increase their expandability while preserving their myogenic potential (Malerba et al., 2008).

MCM was obtained from the J774 murine macrophage cell line (Ralph et al., 1975). Cells were grown in DMEM containing 10% of FBS; when plates were at 90% confluence, after three rinsing in Phosphate Buffered Saline (PBS, 3.2 mM Na_2HPO_4, 0.5 mM KH2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4/0.14 M NaCl, 0.01 M PO_4 Buffer, 0.003 M KCl) cells were re-fed with serum-free medium containing 0.01 µg/ml lipopolysaccharide (*E. coli* LPS, Sigma-Aldrich®). After 24 h the medium was removed and, after thorough PBS rinsing, cultures were kept for 72 more hours in serum-free DMEM. The supernatant was then harvested, centrifuged at 400 ×g for 10 min and passed through a 0.22 µm filter to eliminate the cellular debris and then stored at -20 °C.

Transfection:

Lipofectamine™ 2000, a commercially available cationic lipid formulation from Invitrogen, was used for transfections with AOs. Cultures from pates or flasks were splitted before confluence and ~7×10^4 cells were placed in each well of a 24 multi-well plate.

When cells reached ~90% confluence (and/or first observation of myotubes) growth medium was removed and transfection was carried out in Opti-MEM® I

Reduced Serum Media, without antibiotics (as this reduces transfection efficiency and causes cell death).

LIpoplexes preparation was carried out by mixing 100 µl of a suspension formed by Opti-MEM® and Lipofectamine™ (2 µl per µg of AOs) with a solution of 100 µl Opti-MEM® and the indicated amounts of AOs (indicated quantities are per single well). Both the liposome and DNA solutions were first incubated separately for 5 minutes in ice, then mixed together and left for further 15 minutes in ice. Per each well, 300 µl of Opti-MEM were added, to reach a total volume of 500 µl. Five different AOs amounts were tested (when enough cells were available): 600, 300, 100, 50 and 25 nanomoles per well.

After 24 hours of incubation Opti-MEM® were discarded and total RNA was extracted.

PATIENT IDENTIFICATION NUMBER	TYPE OF MUTATION	EXON TO BE SKIPPED TO RESTORE THE READING FRAME
4969	Deletion exon 51	50
4967	Deletion exons 48-52	53
7547	Point mutation intron 58 (resulting in a deletion of exon 58)	59
8566	Deletion exon 12	11
4445	Deletion exons 46-47	45

Table 1

In this study 2'-O-methyl-phosphorothioate modified AOs were used (Figure 13). AOs were synthesized to anneal to splicing motifs at the intron-exon boundaries, as well as ESE motifs predicted by the web-based application, ESEfinder (Cartegni et al., 2003).

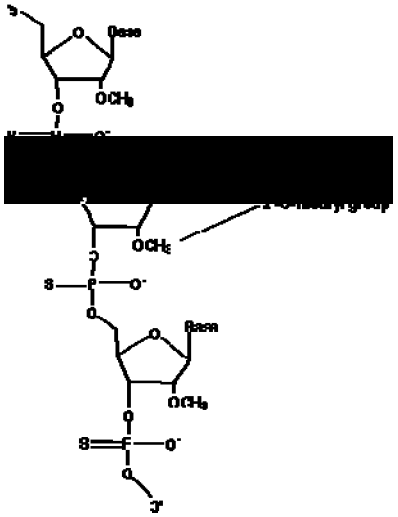

Figure 13 Structure of AOs used in this study, showing backbone and 2' modifications.

Immunofluorescence analysis:

Presence and localisation of dystrophin in DMD cells, transfected cells and wt control cells.

Cells were growth over cover slips and then immunofluorescence analysis was performed with the following protocol:

- Fix in para-formaldehyde (PFA) 2% in PBS, 10 minutes at room temperature.
- Wash once in PBS for 2 minutes.
- Block with PBS containing 3% Bovine Serum Albumin (BSA) for 45 minutes.
- Wash briefly in PBS.
- Incubate with a rabbit polyclonal antibody against dystrophin (Abcam ©), diluted 1:100 in PBS containing 1% BSA, for 60 minutes at 37 °C.
- Wash in PBS three times for 10 minutes.
- Incubate with a Cy™3-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch), diluited 1:200 in PBS containing 1% BSA for 60 minutes at 37 °C.
- Wash in PBS three times for 10 minutes.
- Mount with Fluorescence Mounting Medium (Dako) containing 1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) as nuclear counterstain.

DNA Extraction:

Genomic DNA was obtained performing proteinase K digestion and phenol/chloroform (1:1) extraction.

Cells were treated with lysis buffer (1 ml in a 100mm ø plate containing):

- Na acetate 0.3 M pH 8
- Tris-HCl 10 mM pH 7.8
- EDTA 1 mM pH 8
- SDS 1%

Samples were then treated using the following protocol:

- After adding 5 µl of 20 mg/ml of proteinase K, cell lysate was placed in incubation for 1 hour at 37 °C.
- Tubes were then frozen for 30 minutes at -80 °C.
- Samples still frozen were centrifuged at 12,000×g for 10 minutes at 4 °C and then supernatant was transferred into a new tube.
- An equal volume of phenol/chloroform was added and mixed.
- Centrifugation at 12,000×g for 2 minutes and supernatant transfer.
- Equal volume of chloroform addition.
- Centrifugation at 12,000×g for 2 minutes and supernatant transfer.
- Addition of 1/10 in volume of Na acetate 3 M pH 5.2.
- Addition of an equal volume (of supernatant) of isopropyl alcohol.

- Incubation for 10 minutes at room temperature (RT).
- Centrifugation at 12,000×g for 2 minutes and discard of supernatant.
- Pellet was then washed with ethanol 70%.
- After a short centrifuge at 12,000×g ethanol was removed and pellet was let briefly dry.
- DNA was dissolved in TE (10 mM Tris HCl pH 7.5, 1 mM EDTA)

RNA Extraction:

Total RNA was extracted from cells using TRIzol® reagent. Medium was removed from plates and cells washed once or twice with PBS. Cells were lysed using 250 µl per well. After pipetting, TRIzol® solution was then transferred in a tube and the following steps was followed.

- After 15 minutes of incubation on ice, 0.2 ml of chloroform per ml of TRIzol® were added.
- Tubes were shaken vigorously for 15 seconds.
- After 2 minutes of incubation at RT samples were centrifuged at 12,000×g for 15 minutes at 4 °C.
- After separation, the aqueous phase containing RNA was transferred to a fresh tube.
- 0.6 ml of isopropyl alcohol per ml of TRIzol® were added and mixed thoroughly.
- After 30 minutes of incubation on ice, samples were centrifuged at 12,000×g for 10 minutes at 4 °C.
- Supernatant was removed and pellet was washed once with ethanol 75%.
- After a short centrifuge at 12000×g ethanol was removed and pellet was let briefly dry.
- RNA was dissolved in RNase free water (UltraPure™ DEPC-Treated water).

cDNA synthesis:

Concentration of RNA in the samples (and 260/280 ratio) was calculated using spectrophotometry, then RNA was retrotranscribed using Invitrogen SuperScript™ III Reverse Transcriptase Kit and its standard protocol.

In the reaction ~1 µg of total RNA was used and 250 ng of random hexamers (Promega©) were added as unspecific primers.

A first mix was prepared adding:

- Total RNA
- Primers
- dNTP mix (10 mM each) 1 µl
- RNase free water up to 13 µl

The mix was heated to 65 °C for 5 minutes and then tubes were placed on ice for at least 1 minute. After a brief spin the following reagents were added:

- 4 µl of first-strand buffer 5×
- 1 µl of DTT 0.1 M
- 1 µl (40 units) of RNaseOUT™
- 1 µl (200 units) of SuperScript™ III Reverse Transcriptase

Mix was kept to 25 °C for 5 minutes, followed by 60 minutes at 50 °C; temperature was then increased to 75 °C for 15 minutes to inactivate the enzyme.

The obtained cDNA was then stored at -20 °C if not directly used.

PCR:

Polymerase Chain Reaction was carried out (using primers in Table 2):

The mix for the reaction was:

- DNA sample 1-2 µl
- Buffer 10X Complete 1.25 µl
- dNTP mix 1 mM 2.5 µl
- primers (mix of 10 pM/µl each) 0.5 µl
- AmpliTaq Gold® (Applied Biosystems©) 0.08 µl (0.4 units)
- DEPC-Treated water up to 12,5 µl

PRIMERS FOR RT-PCR

EXON TO TEST SKIPPING	STARTING EXON	FORWARD PRIMER SEQUENCE (5' TO 3')	REVERSE PRIMER SEQUENCE (5' TO 3')	TERMINAL EXON
50	48	CCAAGAAGGACCATTTGACG	CCTCCTTCCATGACTCAAGC	53
53	46	AACCTGGAAAAGAGCAGCAA	CCAGGCAAGAAACTTTTCCA	55
59	56	CCGATGATGCAGTCCTGTTA	ACGTGGCTCACGTTCTCTTT	60
11	9	ACCACCTCTGACCCTACACG	CGTTGCCATTTGAGAAGGAT	14
45	43	GCTCAGGTCGGATTGACATT	GAGTGGCTGGTTTTTCCTTG	49

Table 2

The amplification was performed using the following temperature cycle:

- 94 °C for 12 minutes
- 30-33 cycles with:
  - 94 °C for 30 seconds
  - 60 °C for 30 seconds
  - 72 °C for 45 seconds
- 72 °C for 10 minutes
- 4° C storage

PCR products were then analysed by electrophoresis in agarose gel 0.8-1.6% in TAE 1× buffer (40 mM Tris-acetate, 20 mM sodium acetate, 1 mM EDTA, pH 8.0) containing SYBR® Safe DNA gel stain.

		EXPECTED PRODUCTS' SIZE (bp)			
		PATIENTS		MCQ	
EXON TO TEST SKIPPING	EXONS RANGE	FULL-LENGTH	SKIPPED	FULL-LENGTH	SKIPPED
50	48-53	519	410	752	643
53	46-55	649	437	1397	1185
59	56-60	558	289	679	410
11	9-14	616	434	767	585
45	43-49	749	573	1047	871

Table 3

Band extraction from gel and sequencing:

In order to sequence some of the PCR products, the bands were purified from the agarose gel using the PureLink™ Quick Gel Extraction Kit (Invitrogen™) according to the manufacturers protocol.

Sequencing was carried out by using the DNA extracted from the bands as template for a further PCR reaction (using the same pair of primers). Amplicons were then purified, dried out and sent to BMR sequencing facility.

RESULTS

Transfection set up:

Myoblasts cultures from different patients presented very different morphologies and development rates. In some cases (patients 8566 and 4445, see below) cells showed quite slow proliferation and altered morphology; in these instances we tried to rescue their myogenic phenotype by exposing them to a different medium, specific for culturing satellite cells from single fibers: DMEM, 20% FBS, 10% horse serum and 0.5 % Chicken Embryo Extract (CEE). This approach yielded some improvement in the case of patient 8566 but in the other.

The experimental design with the multiple AO concentrations was modelled after that already in use in Dr. S.D. Wilton laboratory (University of Western Australia, Australian Neuromuscular Research Institute, Centre for Neuromuscular and Neurological Disorders, Perth) with which our group is collaborating in this project.

Transfections:

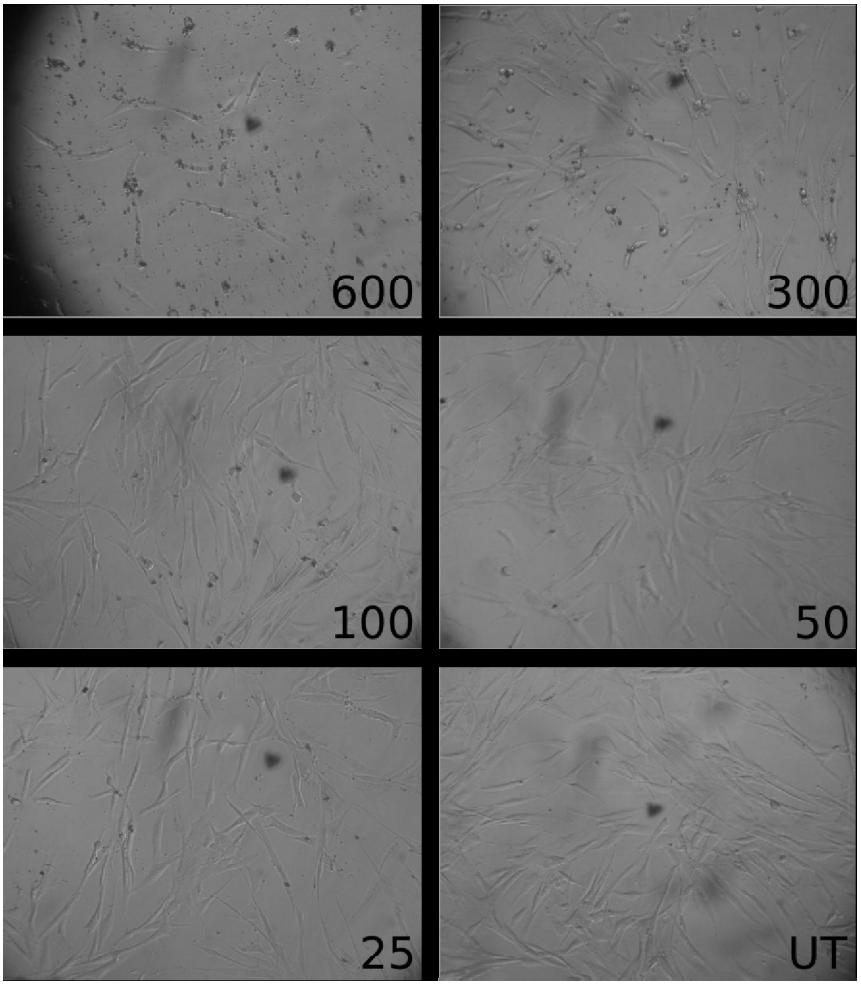

Figure 14 Cells morphology 24 hours after transfection (original magnification 60×).

All cell cultures transfected at confluence of about 90%, a condition that in previous experiments in the laboratory had shown to be well tolerated by primary myoblasts when using the Lipofectamine 2000 reagent with plasmid DNA. In this case, however, 24 hours after transfection, the cells treated with the maximum amount of AO (600 nM) showed signs of sufferance and a lot of them were dead and detached from the bottom of the wells; myoblasts treated with lower quantity of AO showed a morphology similar tothat of the untreated controls (Figure 14).

The nomenclature for naming AOs is the same adopted by the team of Dr. Wilton, which actually synthesized them with their “in house” facility.

Figure 15 illustrates the nomenclature with an example of AO targeting exon 23 of *mdx* mouse.

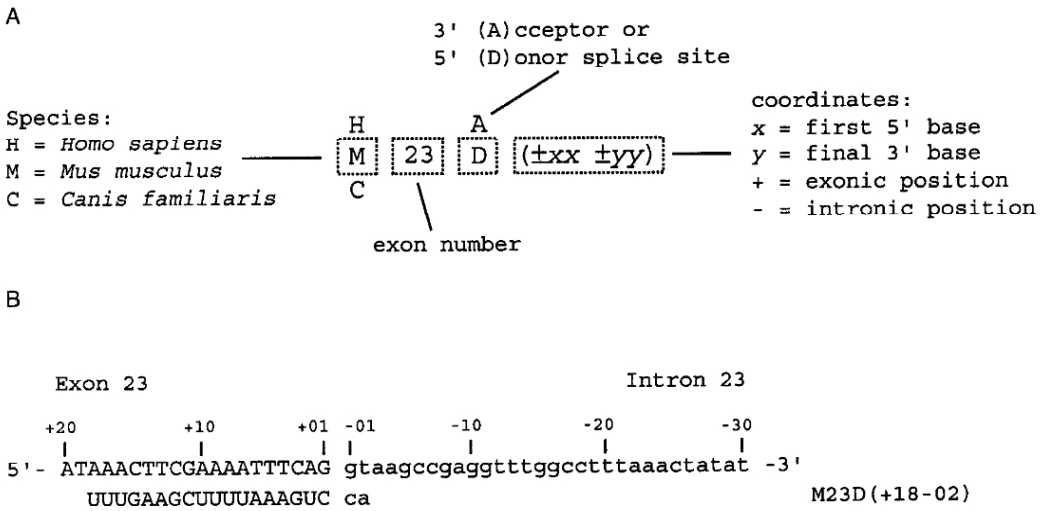

Figure 15 AOs nomenclature adopted.

Test of exon-skipping in wild-type cells:

AO H50A(+01+30)

Target on exon 50:	5' aagAGGAAGT TAGAAGATCT GAGCTCTGAGTGGAAGGCGG TAAACCGTTT ACTTCAAGAG CTGAGGGCAA AGCAGCCTGACCTAGCTCCT GGACTGACCA CTATTGGAGC CTgtaagtat 3'

The PCR primers were positioned in exon 48 (forward) and exon 53 (reverse). This antisense oligo produced a skipping that is consistent with the removal of the target exon: comparison of amplified products size suggest that the lower bands represents an amplified product containing exons from 48 to 53 without exon 50 (643 bp), amplified full-length product (752 bp) is confirmed by the untreated control. In a second transfection with this oligo on MCQ cells, a weak signal of a small band appeared at ~400 bp (compatible with exclusion of all exons between the exons' primers) (Figure 16).

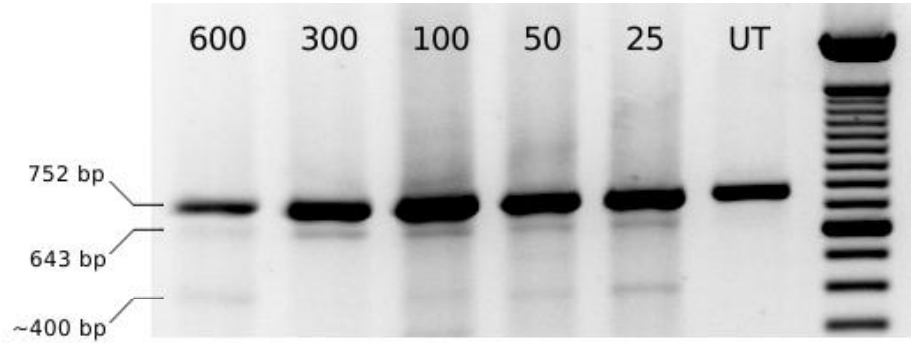

Figure 16

AO H53A(+39+69)

Target on exon 53:	5' tttattctag TTGAAAGAAT TCAGAATCAG TGGGATGAAG TACAAGAACA CCTTCAGAAC CGGAGGCAAC AGTTGAATGA AATGTTAAAG GATTCAACAC AATGGCTGGA AGCTAAGGAA GAAGCTGAGC AGGTCTTAGG ACAGGCCAGA GCCAAGCTTG AGTCATGGAA GGAGGGTCCC TATACAGTAG ATGCAATCCA AAAGAAAATC ACAGAAACCA AGgttagtat 3'

The PCR primers were positioned in exon 46 (forward) and exon 55 (reverse).
The amplification of cDNA, from cells treated with this oligo, showed a subset of shorter amplified products less heavy than the presumed skipping band (1185 bp). Untreated sample confirm the full-length size (1397 bp) (Figure 17).

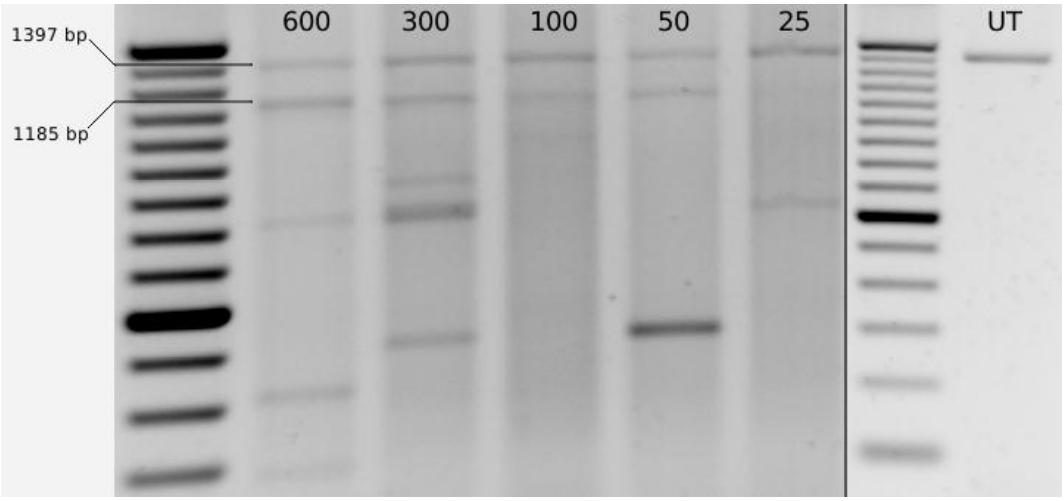

Figure 17

AO H59A(+96+120)

Target on exon 59:	5' agAGCTGCCT CCTGAGGAGA GAGCCCAGAA TGTCACTCGG CTTCTACGAA AGCAGGCTGA GGAGGTCAAT ACTGAGTGGG AAAAATTGAA CCTGCACTCC GCTGACTGGC AGAGAAAAAT AGATGAGACC CTTGAAAGAC TCCgGGAACT TCAAGAGGCC ACGGATGAGC TGGACCTCAA GCTGCGCCAA GCTGAGGTGA TCAAGGGATC CTGGCAGCCC GTGGGCGATC TCCTCATTGA CTCTCTCCAA GATCACCTCG AGAAAGTCAA Ggtaccgtct 3'

The PCR primers were positioned in exon 56 (forward) and exon 60 (reverse).
More than a single skipped product was observed even on the transfection with this AO. The confirmed full-length (679 bp) and the skipped product (410 bp) seems to be present. In the second transfection more alternative skipping were observed (Figure 18).

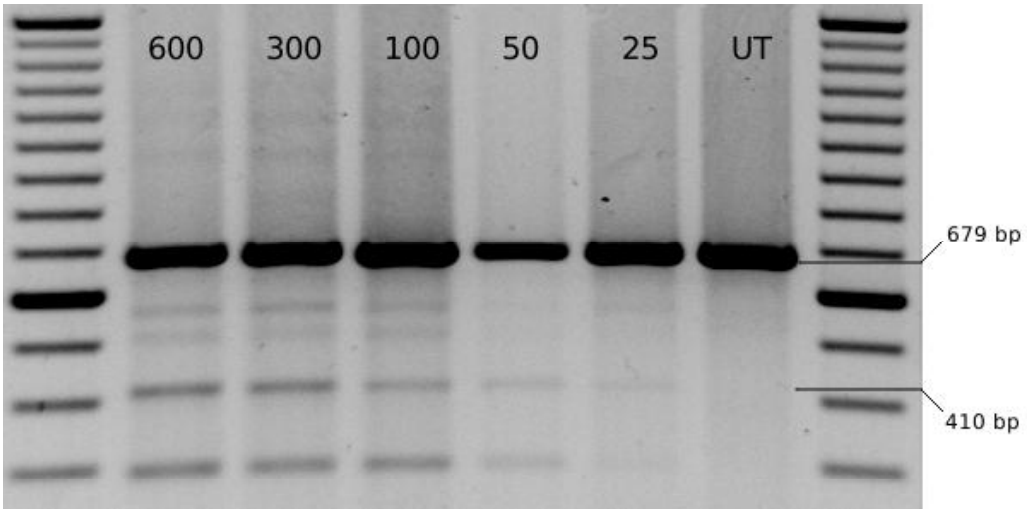

Figure 18

AO H11A(+75+97)

Target on exon 11:	5’ cagGGGTACA TGATGGATTT GACAGCCCAT CAGGGCCGGG TTGGTAATAT TCTACAATTG GGAAGTAAGC TGATTGGAAC AGGAAAATTA TCAGAAGATG AAGAAACTGA AGTACAAGAG CAGATGAATC TCCTAAATTC AAGATGGGAA TGCCTCAGGG TAGCTAGCAT GGAAAAACAA AGCAAgtaag 3’

The PCR primers were positioned in exon 9 (forward) and exon 14 (reverse).
This AO induced skipping compared to the untreated control, however there was two bands distinguishable near the expected skipping product size. A lighter band is observable in the transfections with lower quantity of AO (Figure 19).

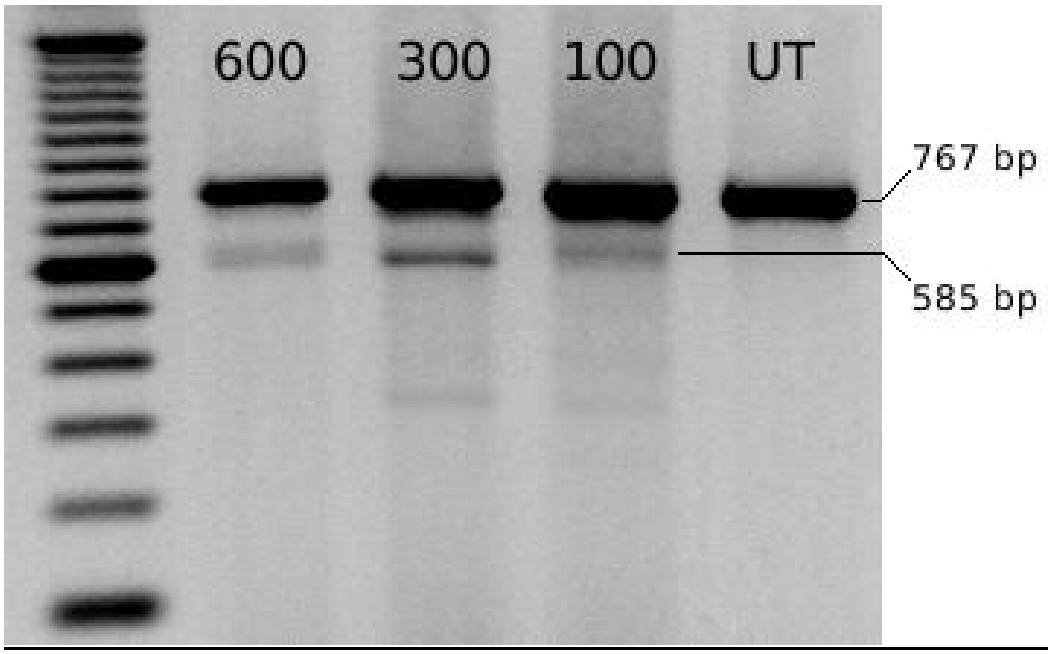

Figure 19

AO H45A(-08+19)

Target on exon 45:	5’ ggtatcttac ag GAACTCCA GGATGGCATT GGGCAGCGGC AAACTGTTGT CAGAACATTG AATGCAACTG GGGAAGAAAT AATTCAGCAA TCCTCAAAAA CAGATGCCAG TATTCTACAG GAAAAATTGG GAAGCCTGAA TCTGCGGTGG CAGGAGGTCT GCAAACAGCT GTCAGACAGA AAAAAGAGgt 3’

The PCR primers were positioned in exon 43 (forward) and exon 49 (reverse).
Transfection with this AO induced skipping consistent with removal of exon 45, however there was another band distinguishable between the expected skipping and the full-length (Figure 20).

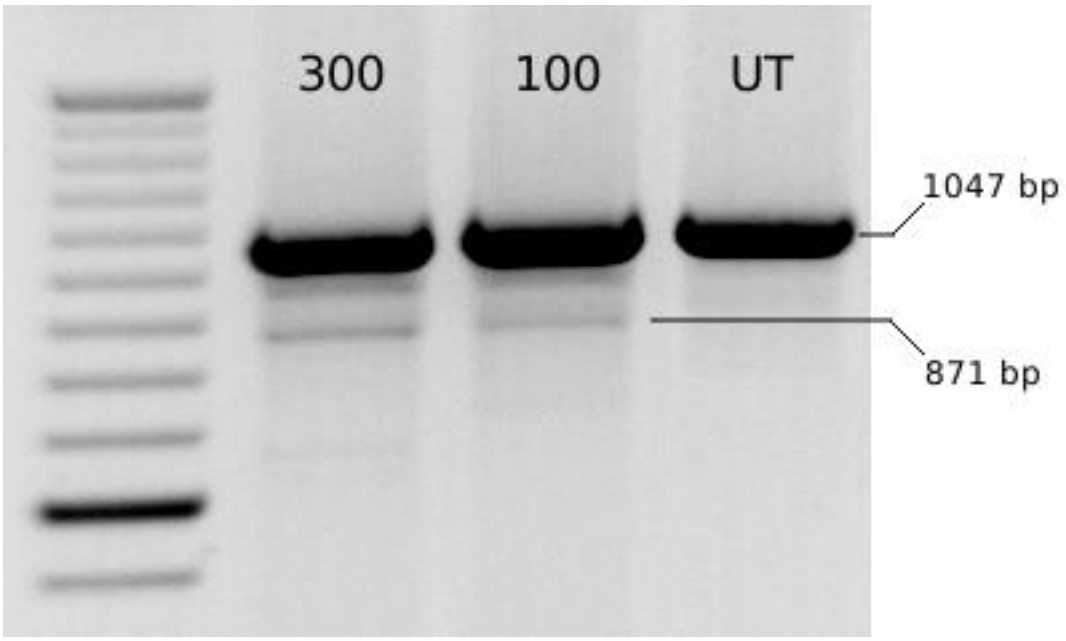

Figure 20

Test of exon-skipping in patients' primary myoblasts:

Patient 4969 – treated with AO H50A(+01+30)

This patient carried a deletion of exon 51 and his cells were therefore transfected with the aim of inducing the skipping of exon 50.

A single skipping product was detected for the myoblasts of this patient (Figure 21).

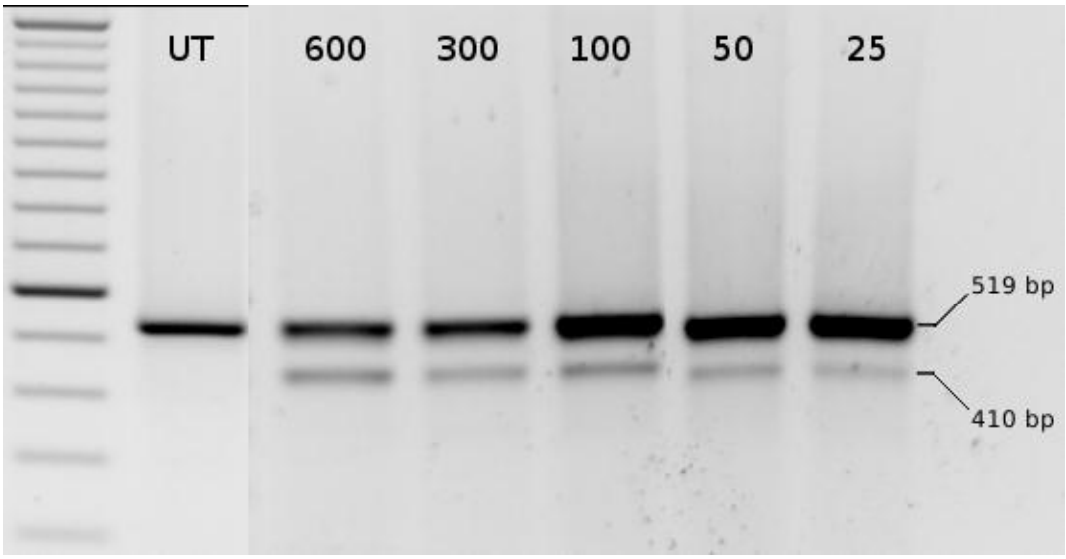

Figure 21

Patient 4967 – treated with AO H53A(+39+69)

This patient carried a deletion of exons 48-52 and his cells were therefore transfected with the aim of inducing the skipping of exon 53.

A single skipping product was detected also for the myoblasts of this patient (Figure 22).

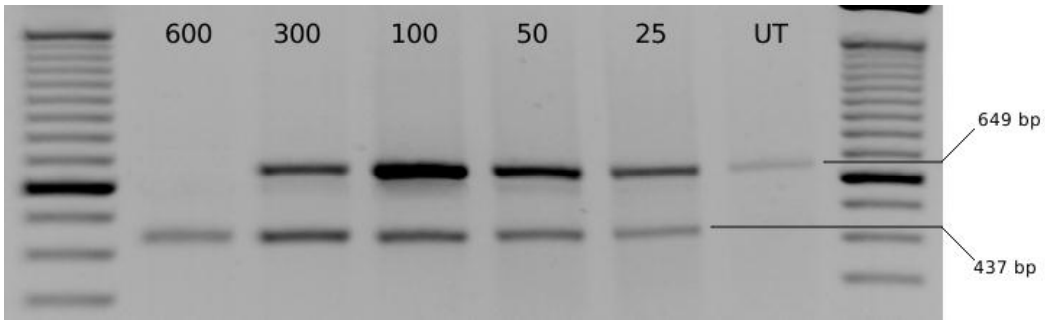

Figure 22

Cells from this patient were also treated over coverslips and investigated for dystrophin expression (Figure 23).

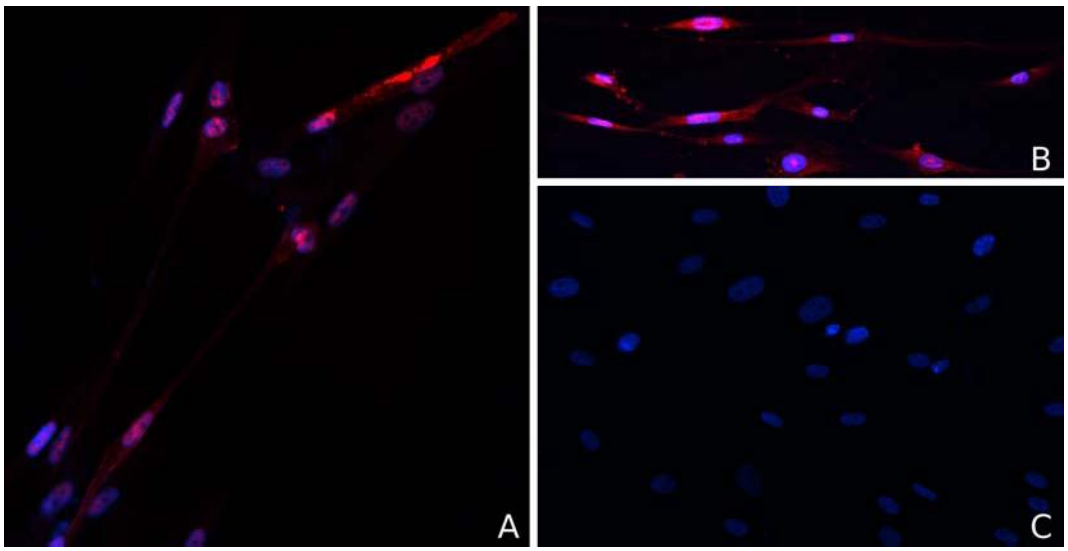

Figure 23 Cells stained for dystrophyn A: MCQ control cells; B: treated cells of patient 4967; C: untreated cells of patient 4967. (Original magnification 40×)

Dystrophin expression is noticeable in treated cells, while untreated controls shown no dystrophin signal.

Patient 7547 – treated with AO H59A(+96+120)

For the purposes of our study cells from this patient were particularly interesting, as the boy had been diagnosed as carrier of a point mutation: c.8668+3 A>T in intron 58 that was confirmed by sequencing exon 58 (Figure 24) using intronic primers targeting outside of the mutation (see Table 4, position of the point mutation shown in bold). Our sequencing confirmed that indeed the mutation report was correct and, importantly, there were no other base changes either in the exon or in the surrounding boundaries.

INTRONIC PRIMERS TARGETING EXON 58
aaattgacct gggagtttca taaacaagtt ctgagcaccc aggattaatt
ttgagaagaa tgccacaagc caaataagca cttcttttca tctcatttca
cagGCCTTCA AGAGGGAATT GAAAACTAAA GAACCTGTAA TCATGAGTAC
TCTTGAGACT GTACGAATAT TTCTGACAGA GCAGCCTTTG GAAGGACTAG
*AGAAACTCTA CCAGGAGCCC AGAGgt**a**att gaatgtggaa ctataataac*
atattgatag aaggatcagt ggtgacggag cagcccatcc attcttgctg

Table 4

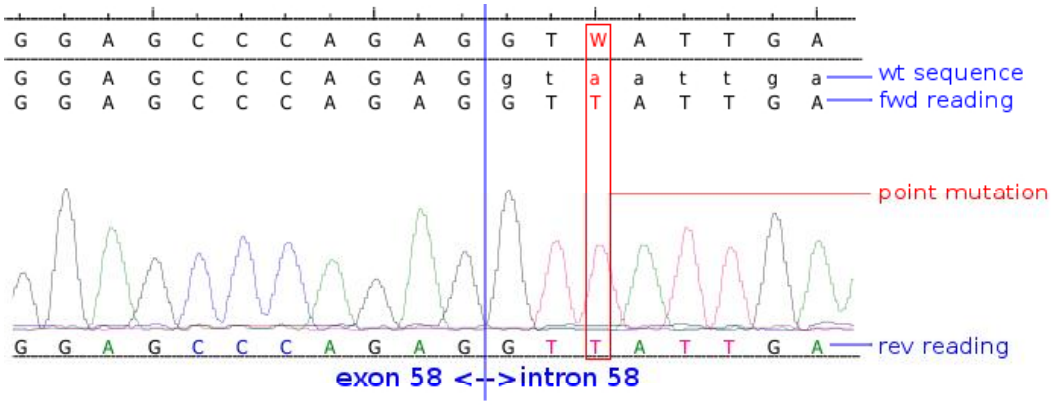

Figure 24 Sequence of point mutation in intron 58.

No RT-PCR analysis had been carried out in this patient, but the fact that he showed a classic DMD phenotype suggested that the most likely cause of disease was the lack of exon 58 in the mature transcript. For this reason we amplified the region 56-60 of its cDNA to verify if this was indeed the case. The amplified product was indeed of the expected size (Figure 25). The deletion was then confirmed by sequencing the band.

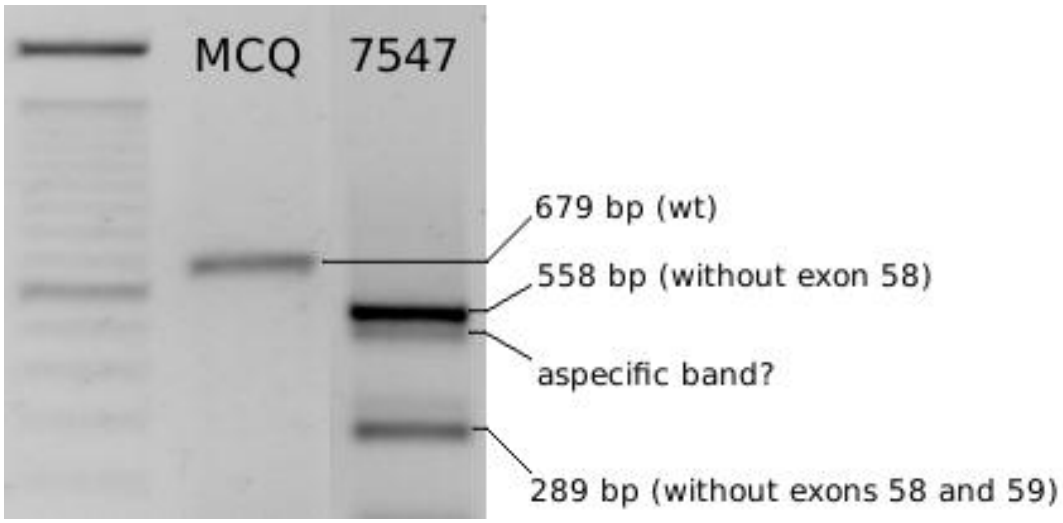

Figure 25

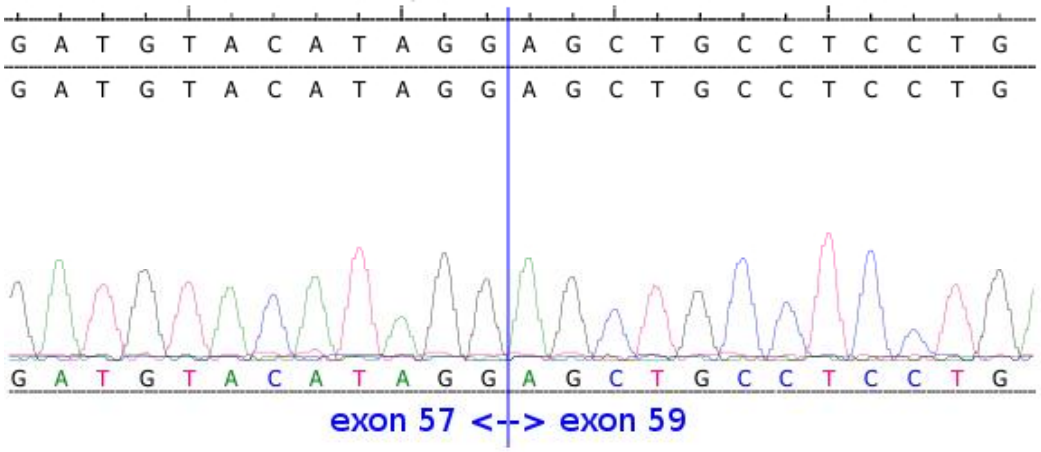

Figure 26 Sequence of the 558 bp amplified product (primers from exon 56 to exon 60) from patient 7547.

It should be noticed that a lighter band (434 bp) was also consistently obtained from the RT-PCR reactions and its length was compatible with the exclusion of exon 59. Later sequencing confirmed this hypothesis (Figure 27).

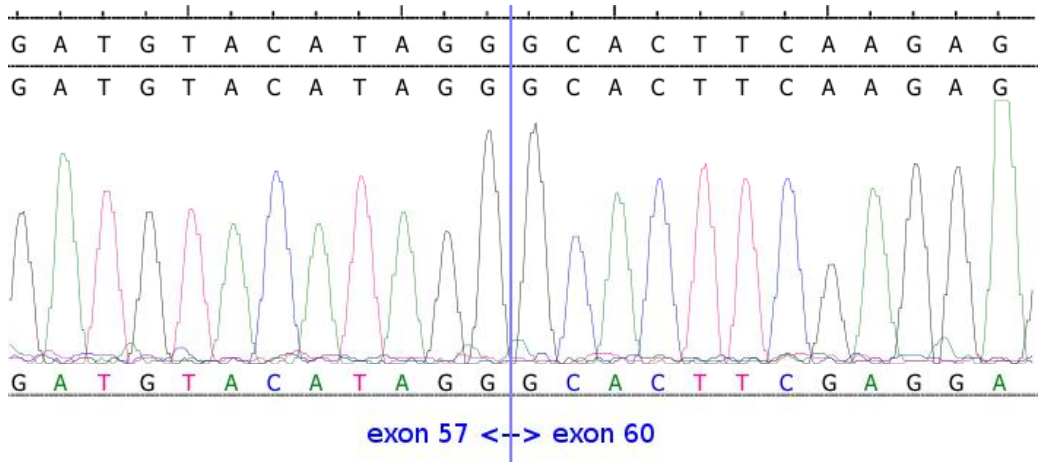

Figure 27 Sequence of the 434 bp amplified product (primers from exon 56 to exon 60) from patient 7547.

This apparently naturally occurring skipping would restore the correct reading frame of the mRNA and hence produce a viable protein.

Treating the cells with the AO aimed at targeting exon 59 we didn't see any change in the resulting transcribed products. Interestingly, however, we did not see any of the extra bands seen with the wild type cells (Figure 29).

Given the fact that even untreated cells appeared to contain a non-marginal amount of in frame transcript we also investigated dystrophin expression in transfected and non-transfected differentiated cultures, but no signal was visible in either cases (Figure 28).

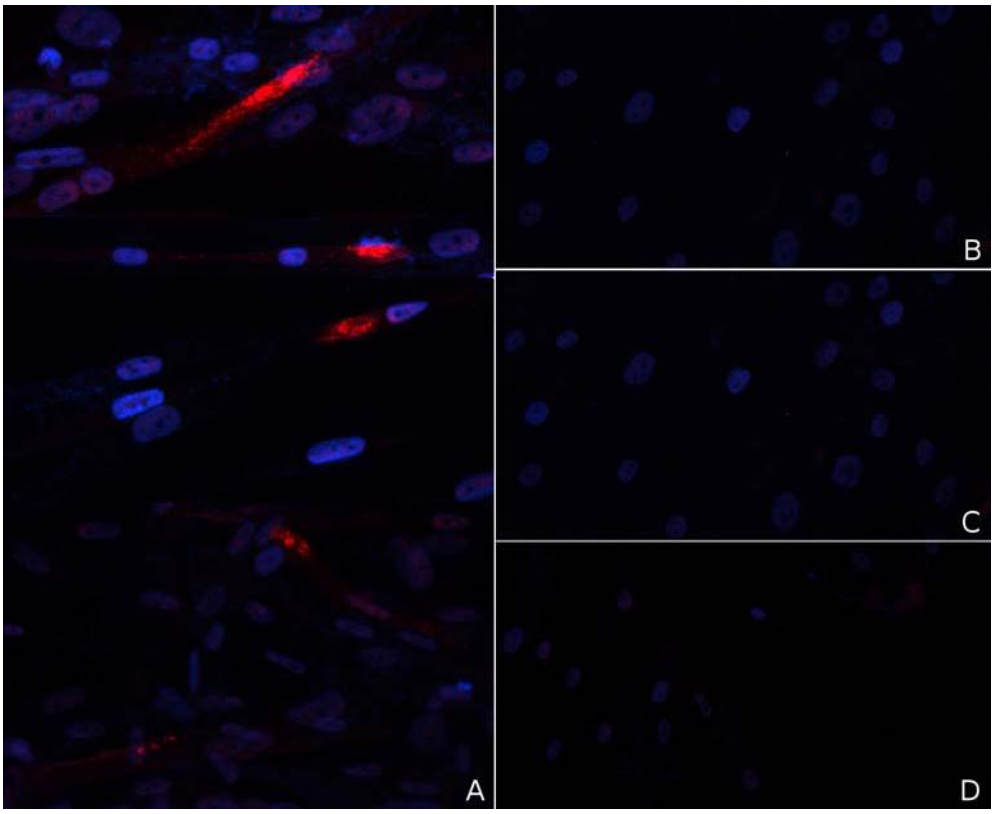

Figure 28 Cells stained for dystrophyn A: MCQ control cells; B and C: treated cells of patient 7547; D: untreated cells of patient 4967. (Original magnification 70× (A) and 40× (B, C and D)).

Acceptor and donor splice site for intron 58 was analysed for the wild type and the mutated sequence: various splicing prediction programs (Table 5) failed to find a putative donor site in the mutated sequence or found a low score similar to the cryptic sites.

USED PROGRAM	SCORE IN WT SEQUENCE	SCORE IN PATIENT SEQUENCE
ASSP (Wang et al., 2006)	9.441	5.220
NNSPLICE (Reese et al., 1997)	0.97	0.43
NetGene2 (Brunak et al., 1991)	0.939	No donor site predicted

Table 5

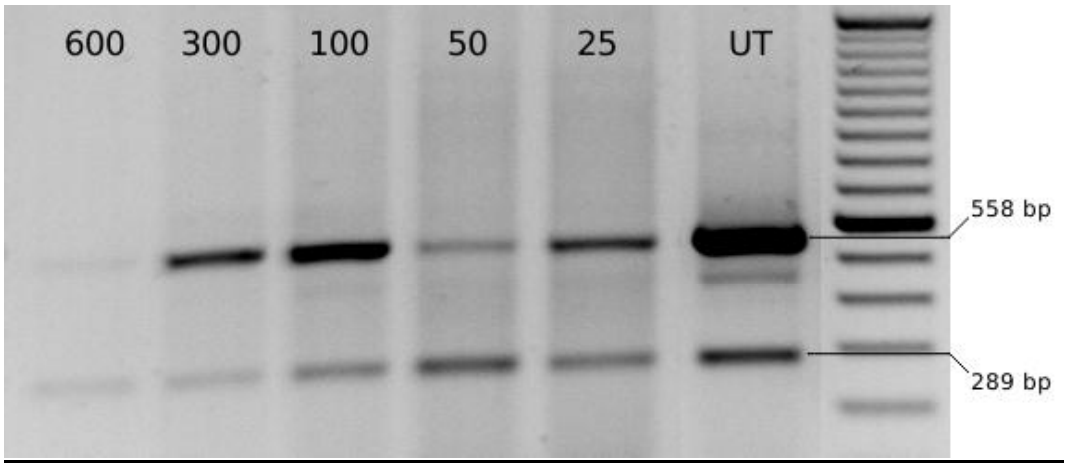

Figure 29

Patient 8566 – treated with AO H11A(+75+97)

This patient carried a deletion of exon 12 and his cells were therefore transfected with the aim of inducing the skipping of exon 11.

Cells from this patient presented a very slow rate of proliferation, even after feeding myoblasts with CEE medium they continued to show normal morphology but very slow mitotic activity. We therefore had only enough cells for three conditions and we decided to transfect them as 300 nM and 100 nM plus the negative control (Figure 30).

Patient 4445 – treated with AO H45A(-08+19)

Myoblasts from this patient suffered a severe contamination, after a treatment with antibiotic/antimycotic, survivor cells presented a "neuronal-like" morphology. We tried to transfect the cells anyway, but the low amount of them permitted to test only one condition plus a control.

Unfortunately RT-PCR failed to amplify from the cDNA (Figure 30).

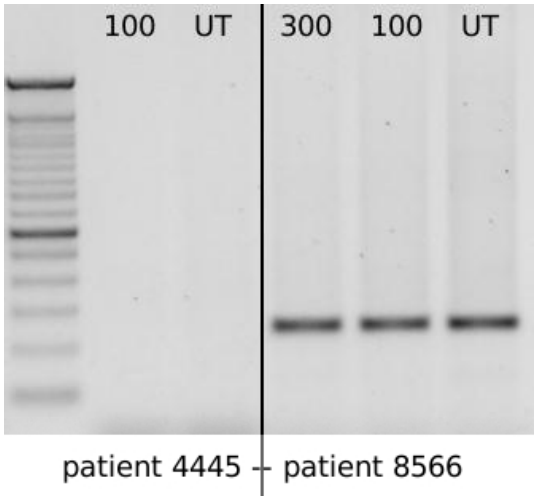

Figure 30

DISCUSSION

The designing of AOs to induce exon skipping for the DMD gene generally follows a multi-step procedure that can be summarized as:

- identify candidate target sequences *in silico*
- test the efficiency of the AOs both on animal models (cell cultures and *in vivo*) and in human cells

Ideally, AOs should be tested on patient's myoblasts but this is often impractical due to the difficulties in accessing this kind of material.
In this work we analyzed the effectiveness of 5 primers, designed to induce the skipping of as many exons, putting them to the test in cultured myoblasts derived from DMD patients and from a normal control.

The H50A(+01+30) oligo, targeted against the exonic region of the splice site, induced the expected exon skipping both in the control and in the myoblasts a patient bearing the deletion of exon 51. However, the skipping amount was generally very low and increasing the amount of oligos used for transfection by more than an order of magnitude made little difference. Hence, this oligo did not appear to be suitable for a possible clinical use.

Control myoblasts transfected with the H53A(+39+69) oligo, directed against a predicted ESE inside exon 53, showed a quite unexpected pattern of alternative splicing in addition to the full-length product and the desired skipping; the pattern consisted of several well-defined bands of smaller sizes. The alternative bands may have derived from exons, other than the target one, excised from the pre-mRNA, or from the use of cryptic splice sites that arose from the interference in the normal splice process. Importantly, the presence of the same alternative skipping products was confirmed in two different experiments. At present we cannot rule out the possibility that some, or even all, of the extra bands were actually non-specific PCR products; however, the fact that they were not found when amplifying the patient's DNA (see below) suggests that they might be derived from the deleted region of the DMD gene.
Myoblasts of a patient (deleted 48-58) were also treated with this AO and showed a unique band representing the expected skipping product, in addition to the full-length found in the treated and untreated controls. This finding hence suggested that the deletion present in the genome of this patient lead to a pre-mRNA without the regions involved in the formation of the various splicing pattern shown in the wild type controls.

The oligo efficiency differed greatly between the two cell types, too, as in the patient it was possible to achieve a near complete suppression of the non-skipped mRNA. Again, this finding could point out at the presence of differences in the splicing process in the two nuclear environments. Further sequencing analyses are planned to determine the identity of the putative alternative splicing products in the control.

Amongst the five patients' cultures available, the most interesting was likely the one derived from a boy who carries an intronic mutation in the acceptor region of intron 58. These cells were tranfected with the H59A(+96+120) oligo and showed a thoroughly unexpected pattern. Indeed, it RT-PCR showed that all samples, including the non transfected control, expressed not only the mRNA without exon 58 but also an alternative splicing product whose size was compatible with the lack of both exons 58 and 59, plus a band of intermediate weight that we could not match to any exon combination. In other words, the in-frame messenger that we were trying to obtain with the skipping turned out to be already present, as sequencing of the band confirmed its identity. Still, the patient in question is a true DMD and immunofluorescence analysis failed to show any signal of dystrophin in his myoblasts, with or without transfection. A quantitative assay to understand the proportion of the natural-occurring skipping will hopefully clarify the situation. At any rate, transfection with our AO did not increase the amount of skipped product, while it reduced the amount of the intermediate band.
When transfecting control cells the AO did induce the formation of the expected skipped mRNA, but also of other bands of different sizes. Given that the intensity of the bands was clearly proportional to the amount of AO used it was unlikely that they were PCR artifacts.
All in all, these findings seem to indicate that our AO is capable of inducing the desired skipping in an intact context (albeit with low efficiency), but it does not work in the particular patient we analyzed.

Another unexpected finding was that provided by the analysis of our Δ 12 patient. In this case cells treated with AO H11A(+75+97) showed a single amplified band – even in the untreated control – that did not match the expected size of either the non-skipped or skipped products. Considering the length of this band it could represent a skipping of all the exons comprised between the primers used for the PCR. The patient's observed splicing is definitely linked to the deletion; in fact, control cells presented the expected PCR products size – both for full-length and skipped product –. In terms of skipping

efficiency the AO did not appear to be very active in the normal genomic context, as even with the highest concentration the amount of skipped product was clearly a small fraction of the full-length product.
Given the problems encountered with this particular batch of cells we are now waiting for another vial of cells from Milan's Telethon biopsy repository in order to carry out more transfection experiments with different primers. At any rate, our first step will be the sequencing of the of patient's amplified cDNA.

Our last primer, AO H45A(-08+19), could only be used on normal cells, as the patient's ones (Δ 46-47) could not be used due to severe contamination of the batch that left us with cells incapable of myogenic differentiation. Control cells could be skipped, but only with low efficiency and also with the presence of an extra intermediate band (between the full-length and the expected skipping product).

In conclusion, the work carried out so far clearly indicates that finding a promising site for inducing exon skipping *in silico* does not necessary means that it will actually work *in vivo*. Programs can predict strength of splice sites and ESE to target, but their algorithms are clearly not fully reliable yet and seem incapable of predicting the interaction between other regions regulating splicing processes and the possible outcome of alternative/cryptic splice sites.
Another important conclusion is that for each AO the skipping efficiency in wild type cells does not necessarily forecast the outcome in a mutated environment. Keeping this result in mind we suggest that further investigations will need to compare patients with the same deletions in terms of missing exons but with different breakpoints. In fact, it will be quite possible that two patients with deleted, say, for 46-47 will require different AO to achieve the skipping of exon 45.
Last but not least, our findings strongly indicated that even apparently "clear cut mutations" like exonic deletions can have unexpected effects at the mRNA level and therefore antisense therapy for DMD patients will have to be a "case by case" approach.

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