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Testing short LNA-modified oligonucleotides for Duchenne Muscular Dystrophy gene therapy

MESTRADO EM BIOLOGIA MOLECULAR E GENÉTICA

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2015

Agradecimentos

Primeiro gostaria de agradecer à Professora Maria Carmo-Fonseca por me ter acolhido no seu laboratório e pelo incentivo dado para que apresentássemos os nossos trabalhos à comunidade científica e pelo *feedback* em relação às nossas prestações em apresentações e no trabalho. Gostaria de agradecer a todos os envolvidos neste projeto, especialmente à minha orientadora, a Doutora Célia Carvalho, por me ter dado a oportunidade de desenvolver este trabalho no Instituto de Medicina Molecular de Lisboa e pela partilha de valiosos conhecimentos, pelas críticas construtivas, pela paciência, pelo apoio e incentivo ao longo do ano que me fez crescer como pessoa e investigadora. Gostaria de agradecer à Professora Doutora Ana Rita Matos pela disponibilidade, apoio, motivação e por ser o meu elo de ligação com a Faculdade de Ciências da Universidade de Lisboa nos aspetos mais burocráticos.

Gostaria de agradecer muitíssimo a todos os colegas do Instituto de Medicina Molecular que me apoiaram no decorrer deste ano. Ao pessoal do Biotério do IMM, gostaria de agradecer a disponibilidade que apresentaram para me ajudar nos momentos de dúvidas e o conhecimento que me concederam, em especial à Dolores Bonaparte pelo ensinamento cuidado na manipulação dos animais. Gostaria de agradecer à Joana Coelho, pela disponibilidade, pelos ensinamentos no que diz respeito a testes funcionais em animais e pela muita paciência que teve comigo e com os ratinhos *mdx52*. Gostaria de agradecer ao Ângelo Chora pela disponibilidade e auxílio prestado na realização das injeções intravenosas nas caudas dos ratinhos *mdx52*. Ao grupo que me acolheu e deu apoio no ano que decorreu, o grupo da Professora Doutora Maria Carmo-Fonseca, um grande obrigado por me receberem, me apoiarem sempre que eu tinha alguma dúvida e me ajudarem de todas as maneiras possíveis. Gostaria ainda de agradecer à Ana Jesus, à Sílvia, à Dinora, ao Sérgio, à Sandra, à Geni e à Noélia pelas muitas discussões e dúvidas que me esclareceram, e à Soraia, Sara, Tomás, Joana Tavares, Catarina, Rita Drago e Rita Mendes de Almeida pelas muitas brincadeiras, pelo apoio e carinho constante e pela motivação que me deram ao longo deste ano. Gostaria de agradecer ao António e Ana Margarida, da Bioimagem, pelos conhecimentos e apoio prestado.

Gostaria de agradecer a todos os meus amigos pelo apoio e pensamento positivo, em particular, à Ana Patrícia, à Joana Oliveira e à Ema pela força e motivação e por acreditarem em mim.

Por último, mas porque os últimos são os primeiros, gostaria de agradecer aos meus pais pelo apoio incansável e constante com que sempre me presentearam e por tornarem possível esta etapa na minha vida.

Resumo

A base hereditária de cada organismo vivo é o genoma, uma longa sequência de ácido desoxirribonucleico (DNA), que contém a informação genética organizada em genes. Em eucariotas, os genes são compostos por exões, interrompidos por intrões. Os exões contêm a informação genética que é traduzida em proteína. Uma vez que a informação genética se encontra no núcleo da célula, e a maquinaria de tradução está localizada no citoplasma, é necessária a formação de um ácido ribonucleico (RNA) mensageiro temporário (pré-mRNA). Este pré-mRNA é processado no núcleo, por uma série de passos que incluem o *capping* da extremidade 5', a remoção de intrões e junção de exões, o splicing e a clivagem e poliadenilação da extremidade 3'. O RNA mensageiro resultante (mRNA) é exportado para o citoplasma, tornando-se disponível para os ribossomas, onde a tradução em proteína decorre.

O *splicing* é um processo altamente complexo e regulado no qual estão envolvidas centenas de proteínas. O *splicing* alternativo, que ocorre quando um único gene origina mais do que uma sequência de mRNA, é um mecanismo importante para a regulação da expressão génica. Uma vez que o *splicing* e o *splicing* alternativo são mecanismos extremamente importantes e conservados na Natureza, a sua disrupção pode levar a doenças. Apesar de a disrupção destes mecanismos estar subjacente a muitas doenças hereditárias e adquiridas, a modulação do *splicing* através da utilização de oligonucleotídeos *antisense* pode ter aplicações terapêuticas. A modulação de *splicing* pode ser conseguida *in vitro* com o uso de compostos químicos ou oligonucleotídeos *antisense* (AONs) que se podem ligar a uma determinada sequência do pré-mRNA e regular o *splicing* do gene, quer para restaurar a função do gene ou para inibir a expressão génica. A modulação do *splicing* oferece esperança para o combate de muitas doenças genéticas, que são atualmente incuráveis. O exemplo mais notável desta aplicação terapêutica é na Distrofia Muscular de Duchenne (DMD), causada principalmente por mutações *nonsense* e de *frame-shift* no gene *DMD*, localizado no cromossoma X, que codifica para a proteína distrofina. As distrofias musculares constituem um grupo heterogéneo de doenças genéticas musculares caracterizadas por um progressivo enfraquecimento, atrofia e degeneração muscular. As distrofias musculares associadas a deficiências no gene da proteína distrofina, podem apresentar vários fenótipos, desde o mais grave e mais comum – a Distrofia Muscular de Duchenne (DMD), até ao mais ligeiro – a Distrofia Muscular de Becker (BMD). A DMD mais severa, apresenta uma incidência de 1 em cada 3500 recém-nascidos do sexo masculino, apresentando a BMD, a mais ligeira, uma incidência de 1 em cada 18 500. A distrofina é a proteína responsável pela manutenção da estabilidade da membrana das fibras musculares. Mutações neste gene levam à perda de função da proteína, sendo esta patologia

caracterizada pela ausência de distrofina funcional no músculo cardíaco, esquelético e liso.

Apesar dos esforços, nenhuma terapia eficaz e clinicamente aplicável foi ainda desenvolvida, sendo no entanto possível atrasar o *onset* da doença recorrendo a terapias com glucocorticoides. Têm sido investigadas terapias genéticas de correção da *reading frame*, sendo as abordagens mais promissoras as que visam o *skipping* de exões com mutações *frame-disrupting* do pré-mRNA, que apesar de introduzirem deleções, conseguem gerar transcritos *in-frame* permitindo a síntese de uma proteína, que mesmo sendo mais pequena, consegue ser funcional. Na terapia desta patologia, o *skipping* de um exão mediado por oligonucleotídeos *antisense* (AONs) pode restaurar a *open reading frame* (ORF) e permitir a síntese de uma proteína parcialmente funcional. O *skipping* de um exão pode ser induzido pela ligação de oligonucleotídeos *antisense*, direcionados para um ou ambos os locais de *splice* ou sequências internas do exão. Teoricamente, a correção da *reading frame* pode ser conseguida com o *skipping* de um ou mais exões que flanqueiem a deleção, ou com o *skipping* de exões *in-frame* que contenham mutações *nonsense*, ou com o *skipping* de exões duplicados. Os oligonucleotídeos *antisense* Drisapersen (com a estrutura química 2'-O-metil) e Eteplirsen (com a estrutura química morfolino) estão atualmente a ser testados em ensaios clínicos de Fase III como terapia para a Distrofia Muscular de Duchenne, e a aguardar aprovação da Agência Europeia de Medicamentos (EMA) e da *Federal Drug Administration* (FDA). Estes oligonucleotídeos *antisense* conseguem restaurar a *reading frame* da distrofina promovendo o *skipping* do exão 51 do gene da distrofina, sendo esta abordagem aplicável a aproximadamente 13% dos pacientes que sofrem desta patologia.

Uma vez que os oligonucleotídeos *antisense*, que têm sido estudados e que avançaram para ensaios clínicos, têm mostrado um sucesso limitado no que diz respeito a eficácia clínica, neste trabalho procuramos testar a utilização de um diferente tipo de oligonucleotídeos: oligonucleotídeos modificados com *Locked Nucleic Acid* (LNA) – LNA-AONs, como nova estratégia para alcançar a correção do gene mutado, resgatando a expressão da proteína distrofina, induzindo o *skipping* direcionado do exão 51. Procurámos descobrir se estes LNA-AONs podem ser utilizados para terapias de modulação de *splicing* com aumento da eficiência. Os nucleótidos LNA possuem uma unidade de açúcar alterada que forma uma ponte metileno. Esta modificação permite propriedades melhoradas em termos de aumento de estabilidade de hibridação com a sequência alvo, de alta sensibilidade, boa descriminação de incompatibilidades (*mismatches*), baixa toxicidade e estabilidade metabólica aumentada, ajustando às propriedades necessárias para terapia humana.

Neste trabalho, linhas celulares derivadas de mioblastos de pacientes com DMD e indivíduos normais (*Mamchaoui, K. et al*, 2011) foram utilizados para indução *in vitro* de *skipping* do exão 51 do pré-mRNA da distrofina e o modelo animal *mdx52* (*Aoki, Y. et al*, 2012), ratinhos que apresentam uma deleção do exão 52 no gene *DMD*, foi utilizado para indução *in vivo* de *skipping* do exão 51 do pre-mRNA da distrofina nos tecidos musculares. Pesquisámos *skipping* do exão 51 ao nível dos transcritos, através da utilização de técnicas de RT-PCR e pesquisámos a restauração da produção da proteína, através da utilização de técnicas de Western Blot e Imunofluorescência. Os nossos resultados mostraram *skipping* eficaz do exão 51 e restau-

ração da produção da proteína distrofina em mioblastos distróficos de pacientes, transfectados com oligonucleotídeos modificados com LNA utilizando concentrações tão baixas como 5nM. No modelo animal, o ratinho *mdx52*, após cinco injeções intravenosas na cauda, a cada duas semanas, com 1mg/kg de LNA-AON, duas semanas após a última injeção, não foi detetado *skipping* do exão 51 por RT-PCR, nem proteína via Western Blot. No entanto, aglomerados de fibras positivas para a distrofina foram detetadas por imunohistoquímica em ratinhos *mdx52* tratados,e não em ratinhos injectados como controlo, levando a crer que são fibras em que a produção de distrofina foi induzida terapeuticamente devido ao LNA-AON usado. Ocasionalmente, observamos a presença de fibras positivas isoladas para a distrofina em ratinhos não tratados, no entanto nestes casos estamos perante fibras revertentes, ou seja, fibras isoladas ocasionais que ocorrem naturalmente e parecem expressar distrofina corretamente localizada. Estas fibras revertentes poderão ser exemplos onde, por acaso, mutações secundárias adicionais ou eventos intrínsecos aberrantes de *splicing* proporcionam o *skipping* de um ou mais exões adicionais de forma a restaurar a *reading frame* correta original, permitindo a produção de uma proteína funcional. Para que ocorra uma reversão do fenótipo da DMD para um fenótipo menos severo, como o da BMD, ou para evitar distrofias musculares em humano e ratinho, é necessária a expressão de níveis de 20 a 30% da distrofina normal no tecido muscular.

O objetivo deste projeto foi testar se LNA-AONs poderiam ser utilizados para terapias de modulação *splicing* com maior eficiência, em relação aos AONs já estudados. Nós demonstramos que o resgate de expressão de distrofina realizando o *skipping* do exão 51 é viável em linhas celulares derivadas de mioblastos *in vitro*, e em ratinhos *mdx52* distróficos *in vivo*. Os resultados apresentados, obtidos com o modelo celular, parecem muito promissores, a fim de alcançar uma boa recuperação da proteína distrofina em mioblastos de doentes DMD bem como os alcançados em em ratinhos *mdx52*. Seguidamente será necessária otimização do sistema de entrega dos oligonucleotídeos para o tecido muscular de forma generalizada, uma vez que o tratamento de todo o organismo é um desafio e que os tecidos envolvidos são pós-mitóticos, sendo aproximadamente 30 a 40% do corpo humano constituído por músculo. A melhoria da eficiência de modulação de *splicing* e da biodistribuição de oligonucleotídeos *antisense* (AONs) pode reduzir a dose terapêutica e intervalo das administrações, minimizando a potencial toxicidade, efeitos *off-target*, e custos. Com este trabalho mostrámos a aplicabilidade de pequenos oligonuleótidos LNA modificados para terapia genética da Distrofia Muscular de Duchenne, abrindo caminho para uma pesquisa de métodos mais eficientes para terapia genética por modulação de *splicing* em tratamento sistémico de uma doença genética hereditária.

Palavras-chave: Distrofia Muscular de Duchenne, Terapias de Modulação de Splicing, Oligonucleótidos modificados com LNAs, Skipping de exões, Splicing

Abstract

Genetic disorders are caused by abnormalities in an individual's DNA. Novel therapeutic strategies for this types of diseases have been emerging, especially on therapies that involve the modulation of disease-related gene expression. Modulation of splicing using antisense oligonucleotides (AONs) is feasible *in vitro* and can have possible therapeutic applications.

The most notable example is in Duchenne Muscular Dystrophy (DMD), a genetic hereditary disease caused mainly by frame-shifting or nonsense mutations in the DMD gene in chromosome X, which encodes for the dystrophin protein, essential for membrane stability of muscle fibers. In DMD cells, antisense-mediated exon skipping can restore the open reading frame and allow synthesis of partly functional dystrophin proteins instead of non-functional proteins, transforming DMD in the milder Becker Muscular Dystrophy (BMD). The antisense oligonucleotides Drisapersen (2'-O-methyl chemistry) and Eteplirsen (morpholino chemistry) are currently being tested in clinical trials as a therapy for DMD. These aim to restore the dystrophin reading frame by promoting skipping of exon 51, an approach that is applicable to approximately 13% of DMD patients. Locked Nucleic Acid (LNA) modified oligonucleotides carry an altered sugar moiety that forms a methylene bridge allowing improved properties in terms of increased duplex stability, high sensitivity, good mismatch discrimination, low toxicity and increased metabolic stability, fitting the properties needed for human therapy. An oligonucleotide wuth this chemistry is currently awaiting for clinical application as HCV infection therapy after good results in clinical trials. We aim to test if LNA oligonucleotides (LNA-AON) can be used for splicing modulation therapies, with increased efficiency.

In this work, myoblast derived cell lines from patients and *mdx52* mice were used for *in vitro* and *in vivo* induction of skipping of DMD-exon 51, respectively. We looked for skipping of exon 51 at the transcript level (RT-PCR) and for restoration of protein production (Western Blot and Immunofluorescence). Our results show effective skipping of exon 51 and restoration of dystrophin protein production in dystrophic myoblasts transfected with the LNA-AON at concentrations as low as 5nM. In the animal model, the *mdx52* mouse, after five biweekly tail intravenous injections of 1mg/kg with the LNA-AON, we were able to visualize clusters of dystrophin positive fibers therapeutically induced by the LNA-AON on cryosections of selected muscles.

With this work we showed the applicability of short LNA-modified oligonucleotides for Duchenne Muscular Dystrophy gene therapy, paving the way for a search of more efficient methods for gene therapy splicing modulation in systemic treatment of an inherited genetic disease.

Keywords: Duchenne Muscular Dystrophy, Splice Modulation Therapy, LNA Oligonucleotides, Exon Skipping, Splicing

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Introduction

Genetic disorders are caused by abnormalities in an individual's DNA. Novel genetic therapeutic strategies for this types of diseases are emerging. We will focus especially on therapies that involve the modulation of disease-related gene expression, since pre-mRNA splicing is an essential step for eukaryotic gene expression[1]. The modulation of splicing can be achieved *in vitro* with the usage of chemical compounds or antisense oligonucleotides (AONs) that can bind to a target site in pre-mRNA and interfere with RNA splicing, either to restore gene function or to inhibit gene expression[1]. Splicing modulation offers hope for combatting many genetic diseases that are currently untreatable. Different approaches have been developed with the aim of treating some genetic disorders like ataxia-telangiectasia, methylmalonic academia, dystrophia myotonica[1]. The disease in which research on a splicing modulation therapy is more advanced is Duchenne Muscular Dystrophy (DMD).

Splicing Mechanics

The hereditary basis of every living organism is the genome, a long sequence of deoxyribonucleic acid (DNA), that contains the genetic information organized in genes[2]. In eukaryotes, the genes are composed of exons and are interrupted by introns[2]. The exons contain the genetic information that is translated by the cell into protein, while the introns do not contain protein encoding information[2,3,4]. Since the genetic information is enclosed in the cell nucleus, and the translating machinery is located in the cytoplasm, a temporary messenger ribonucleic acid (RNA) molecule (mRNA) needs to be generated[4]. The pre-mRNA is processed in the nucleus, by a series of steps that include 5' end capping, the removal of introns and joining of exons, splicing and 3' end cleavage and polyadenylation[2,4,5,6], resulting in a mRNA that is exported from the nucleus to the cytoplasm, becoming available to the ribosomes where translation into protein ensues[4,6] (Figure 1).

Splicing is a highly complex, tightly regulated, process in which hundreds of proteins and RNAs are involved[3,4,5]. Splicing requires four loosely defined sequence elements in the pre-mRNA, which are the 5' splice site (consensus in mammals: AG/GURAGU), the branch point (YNYURAC), a variable stretch of pyrimidines termed polypyrimidine tract and the 3' splice site (YAG/N; where "/" denotes the exon-intron boundary)[2,7]. The splice sites are sequences immediately surrounding the exon-intron boundaries that include the sites of breakage and reunion of exons[2]. The initial process of splice site recognition, by sequence motifs in introns and exons, commits the pre-mRNA substrate to the splicing pathway and are important for proper processing of pre-mRNA into mRNA[2]. The 5' and 3' splice sites and the branch point sequences are

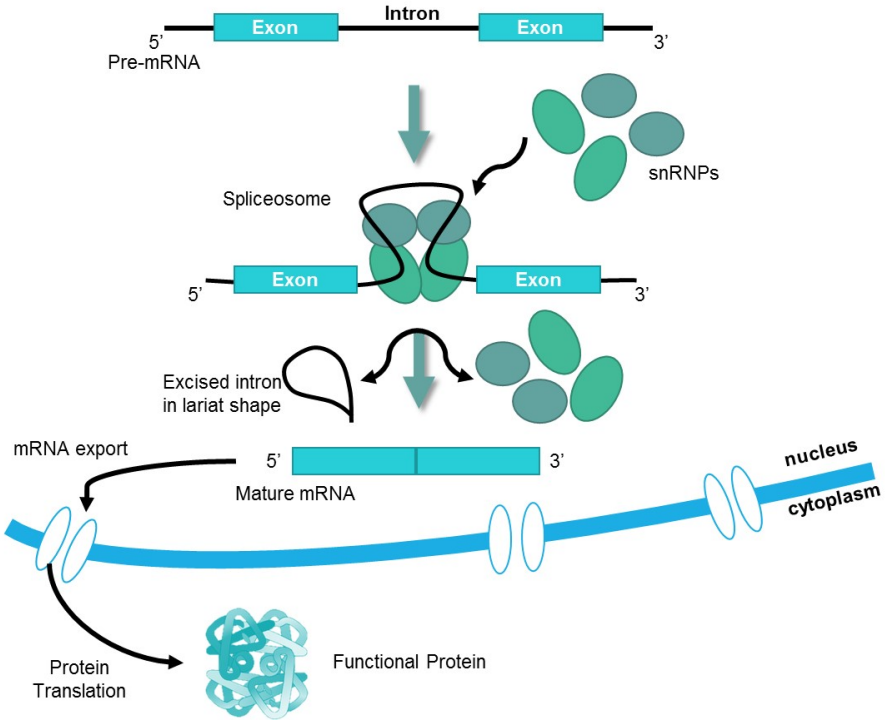

Figure 1: Schematic representation of the splicing process.

recognized by components of the splicing apparatus that assemble to form a large complex – the spliceosome[2]. The splicing reaction proceeds via two sequential transesterification reactions: a lariat is formed when the intron is cleaved at the 5' splice site and the 5' end is joined to a 2' position at the adenosine (A) at the branch site in the intron, the intron is released as a lariat when it is cleaved at the 3' splice site, and the left and right exons are then ligated together[2]. Since introns can be very large in vertebrates, recognition of the splice site involves additional interactions across the exon between the 3' splice site and the downstream 5' splice site, this process is known as exon definition[2].

When a gene gives rise to a single type of spliced mRNA, there is no ambiguity in assignment of exons and introns, however, the vast majority of mammalian genes are spliced and follow patterns of alternative splicing[4,2,5,8]. Alternative splicing occurs when a single gene gives rise to more than one mRNA sequence, and explains how a huge proteome (>100,000 proteins) can arise from a relatively small number of genes (25,000 on the human genome), being also an important mechanism for regulation of gene expression[4,2,5,8]. There are various modes of alternative splicing, including intron retention, alternative 5' splice site selection, alternative 3' splice site selection, cassette exon inclusion or skipping and mutually exclusive selections of the alternative exons[2,5,8].

Alternative splicing is often associated with weak splice sites, meaning that the splicing signals located at both ends of introns diverge from the consensus splicing signals[2]. The sequences surrounding alternative exons are often more evolutionary conserved than sequences flanking constitutive exons[2]. The regulation of alternative splicing is a complex process that involves a large number of alternative splicing regulators[2]. This alternative splicing regulators

may recognize RNA elements in specific exonic and intronic sequences, near the splice site, and enhance or suppress splice site selection[2]. The exonic alternative splicing sequences that enhance splice site selection are referred to as exonic splicing enhancers (ESEs)[2]. When the exonic sequences promote suppression of splice site selection, these are called exonic splicing silencers (ESSs)[2]. Similarly, many elements can affect splice site selection through intronic sequences, being referred to as intronic splicing enhancers (ISEs) and intronic splicing silencers (ISSs)[2]. The effect of splicing enhancers and silencers is mediated by sequence-specific RNA binding proteins, many of which may be developmentally regulated and/or expressed in a tissue-specific manner[4,2,5,8]. Since splicing and alternative splicing are such important and well conserved mechanisms, in their disruption underlie many inherited and acquired genetic diseases[4,5,8,9].

Modulation of splicing can be achieved *in vitro* by oligonucleotides containing sequences complementary (antisense) to unique sequences within the pre-mRNA, which can bring about the exclusion or inclusion of an exon, modifying splicing and, thus, gene expression[10,5,6,11]. This has potential therapeutic applications and antisense-mediated splicing modulation to block aberrant splice sites, to correct disrupted alternative splicing levels, to include aberrantly skipped exons and to induce exon skipping to knockdown protein levels are currently targets of intensive research[4,11,10,12]. The most notable example of this approach is in Duchenne Muscular Dystrophy (DMD), where an antisense-mediated exon skipping therapy is currently finishing phase III trials and awaiting FDA and EMA approval and viewed as the most promising approach to allow treatment of this incurable disease in the near future[10,4].

Duchenne Muscular Dystrophy (DMD)

Duchenne Muscular Dystrophy (DMD) is an inherited X-linked recessive allelic disorder[13]. DMD, the most common form of muscular dystrophy, with an incidence of 1 in 3500 newborn males, is characterized by progressive deterioration of muscle function, with most patients not living beyond age of 30 due to cardiac and respiratory complications[14,13]. This disease occurs mainly due to frame-shifting deletion or nonsense mutations in the *DMD* gene that encode for the dystrophin protein, and comprises 79 exons that produce the longest primary transcript[15,13]. Another *DMD* gene disorder is Becker Muscular Dystrophy (BMD), with an incidence of 1 in 18500 newborn males, presents a large spectrum of severities, from borderline DMD to almost asymptomatic cases[13,16]. In DMD, the disruption of the reading frame leads to the absence of functional dystrophin protein[15], while BMD typically results from shortened but in-frame transcripts of the *DMD* gene that allow expression of an internally truncated but partially functional protein[13,16,17] (Figure 2). Chamberlain determined how much dystrophin was needed to prevent dystrophic pathology in transgenic mice (20-30%) and explored the percentage of muscle fibers that would need to be converted to a dystrophin positive phenotype to achieve a substantial correction of the pathology[18,19,20]. Their results indicated that a majority of fibers must accumulate approximately 20% of wt levels of dystrophin for a significant correction of the muscle pathology[18].

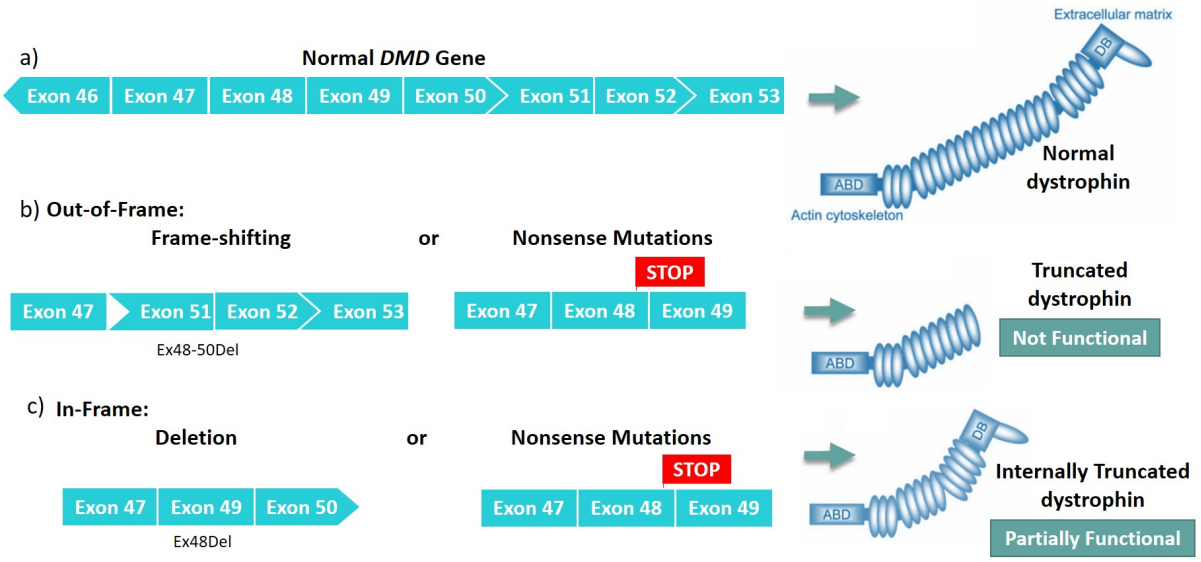

Figure 2: Differences between a normal dystrophin protein (a) and the dystrophin produced in Duchenne patients (b) or Becker patients (c).

In muscle cells, the dystrophin protein associates with numerous proteins (dystroglycans, sarcoglycans, sarcospan, α-dystrobrevins, syntrophins, syncoilin and others) to form the dystrophin associated protein complex (DAPC)[21]. The DAPC plays a structural role in maintaining muscle integrity, stabilizing the sarcolemma during repeated cycles of contraction and relaxation, and transmitting force generated in the muscle sarcomeres to the extracellular matrix[21,13]. The dystrophin, via the transmembrane dystroglycan protein and its associated protein complex, including the sarcoglycans, is able to link the actin cytoskeleton to the extracellular matrix[13] (Figure 3). The muscle isoform of dystrophin is a 427kDa protein that binds to cytoskeletal actin via its N-terminal actin-binding domain 1 (ABD1) and to β-dystroglycan via its C-terminal domain, with the central rod domain, consisting of 24 spectrin-like repeats, in between[21,13]. In order to be functional the dystrophin protein requires its N- and C-terminal domain and much of the rod domain appears to be partially dispensable (at least 8 integral repeats are needed for functionality), dystrophin deficiency leads to the loss of the associated protein complex[21]. In the absence of dystrophin, the muscle membrane becomes susceptible to damage and muscle fiber deterioration occurs, resulting in cycles of regeneration that lead to replacement of muscle fibers by fibrotic or adipose tissues, with the subsequent loss of muscle fibers and muscle function[13,4].

In DMD, the mainstays of therapy are glucocorticoid corticosteroids (prednisone and deflazacort) and palliative care (respiratory support and management of cardiac complications), which slow down disease progression, but do not prevent the progressive loss of muscle fibers and muscle function with increasing disability[13,19,10].

To date there is no cure for this disease, however novel therapies for DMD have already started to arise towards a variety of gene corrective, gene replacement and surrogate gene approaches[13]. Since this disease results from mutations in a single gene, gene therapy to replace the defective gene is a very attractive approach[10]. However, a major challenge for therapy of

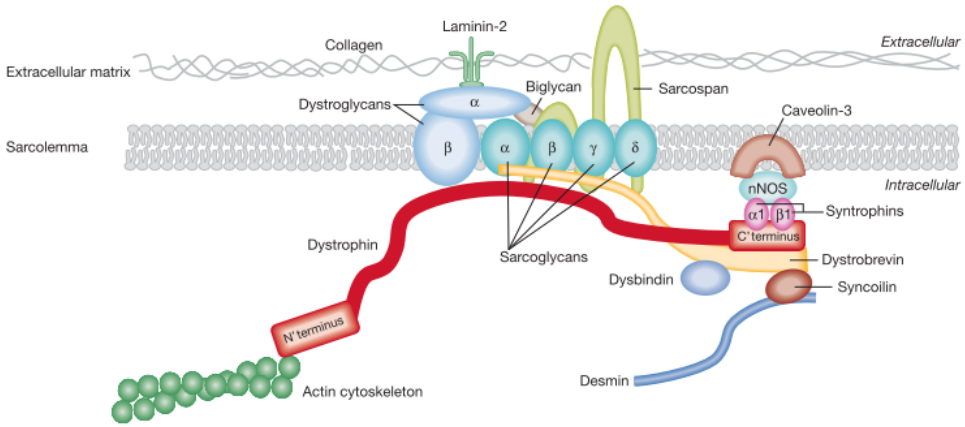

Figure 3: The dystrophin-associated protein complex in muscle linking the internal cytoskeleton to the extracellular matrix[22].

DMD is the need to devise a treatment strategy that targets whole-body musculature, including limb muscles, respiratory muscles (intercostal and diaphragm muscles), cardiac muscle and smooth muscle of the gastroesophageal tract, being this approach generally referred to as systemic therapy[19,10]. Whole-body treatment is particularly challenging in DMD because the tissues involved are post-mitotic and approximately 30 to 40% of the human body consists of muscle[10]. Although adeno-associated virus (AAV) is one of the few viral vector systems that efficiently infects muscle, it has a small cloning capacity that is easily exceeded, since the DMD gene presents 79 exons that correlate with a 13,993bp transcript[10,13]. Thus, it is not surprising that research also focuses on ways to restore gene expression at the mRNA level, more specifically by modulating its final presentation[10].

Antisense Oligonucleotides (AONs) and Splice-Switching Oligonucleotides (SSOs)

Targeting splicing by antisense oligonucleotides (AONs), small synthetic RNAs, DNAs or analogs, which hybridize specifically to their target sequences, allows RNA modifications that are not possible with RNA interference or other antisense techniques that destine the RNA for destruction[10,23,24,25]. The appeal of targeting RNA is easy to appreciate: the nucleotide sequence provides an opportunity to design sequence-specific and therefore gene-specific drugs[24]. AONs can cause inhibition or redirection of splicing and inhibition of protein synthesis through various mechanisms, including disruption of the cell's splicing machinery, interference with the ribosomal complex, and/or by activation of RNase H1-mediated degradation of the oligo-RNA heteroduplex[26,27,25]. Splicing modulation is accomplished by the use of AONs, termed splice-switching oligonucleotides (SSOs), which aim to modify the splicing pattern of a pre-mRNA[24,25]. These SSOs target nuclear pre-mRNA molecules to change exon splicing and generate an alternative protein isoform[28]. SSOs were first described for correction of aberrant splicing in human β-globin pre-mRNAs[29], but have progressed furthest in the research for a treatment of DMD[28].

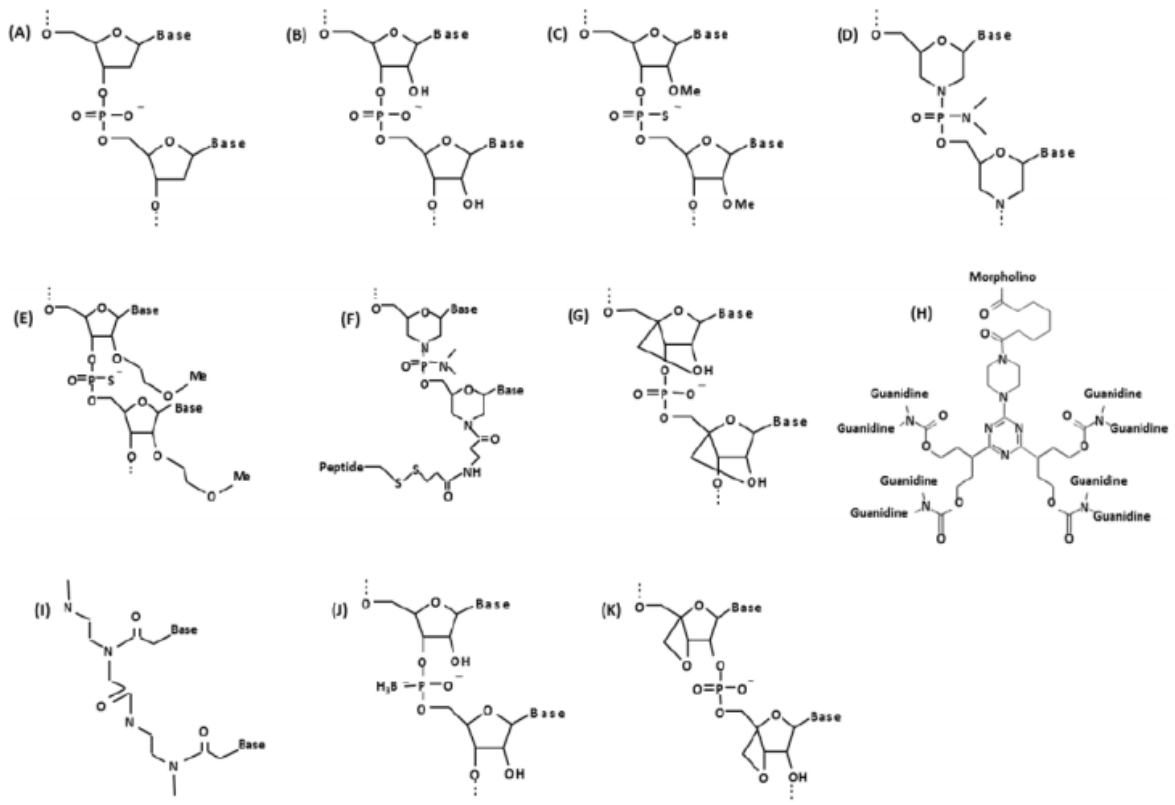

Figure 4: **Chemical structure of biological and synthetic oligonucleotides.** (A) DNA; (B) RNA; (C) 2'O-methylphosphorothioate (2'O-MePS); (D) Morpholino (PMO); (E) 2'-methoxyethoxy (2'-MOE); (F) PMO with peptide conjugate (PPMO); (G) Locked Nucleic Acid (LNA); (H) Vivo-morpholino (vPMO); (I) Peptide nucleic acid (PNA); (J) Boranophosphate-oligodeoxy-nucleoside (BH3-ODN); (K) Oxetane-modified AONs[26].

The first hurdle that first generation AONs had to overcome was regarding drug delivery[26]. Since these AONs do not easily cross the lipid bilayer of the cell membrane, they cannot readily penetrate to their intracellular targets at significant concentrations to be effective[26]. Another problem associated with first generation AONs is off-target toxic effects, because DNA and RNA can be immunostimulatory, binding and activating receptors involved in innate immunity in a sequence- and chemistry-dependent manner[26]. Furthermore, to achieve biochemical efficacy, a large proportion of RNA targets must be hybridized and silenced[26]. In order to overcome these challenges, new AONs have been conceived such that the ribose backbone (normally present in RNA and DNA) is replaced with other chemistries[26]. A variety of AON chemistries have been developed (Figure 4), which can be so distinct from classical nucleic acid structures that are not anymore targeted by nucleases or DNA/RNA-binding proteins, hence avoiding nuclease degradation, facilitating stronger base-pairing with target mRNA sequences, increasing stability, helping to prevent most off-target toxic effects, and enabling easier entrance into the cell[26].

Antisense-mediated Exon Skipping: Applicability in DMD

Antisense-mediated exon skipping aiming for reading frame restoration for DMD is a mutation specific approach and so a personalized therapy. However the majority of DMD patients have deletions that cluster in hotspot regions, being the skipping of a small number of exons applicable to a relatively large number of patients[30]. In theory, single and double exon skipping would be applicable to 79% of deletions, 91% of small mutations, and 73% of duplications, amounting to 83% of all DMD mutations[30]. Exon 51 skipping, which is currently being tested in clinical trials, would be applicable to the largest group (13%) of all DMD patients[30] (Table 1). However, 17% of DMD patients, which carry larger deletions (>36 exons) or deletions in the actin-binding N-terminus or the C-terminus of dystrophin are not eligeble to be treated by a exon skipping approach[30,31].

Table 1: Summary of the Exon-Skipping Applicability in DMD Patients[31,30].
DMD deletions were reported in the Leiden DMD mutation database (www.dmd.nl/).

Exon to Skip	Therapeutic for DMD deletions (exons)*	Applicability (%)
2	3-7	1.9
8	3-7, 4-7, 5-7, 6-7	2.3
43	44, 44-47	3.8
44	35-43, 45, 45-54	6.2
45	18-44, 44, 46-47, 46-48, 46-49, 46-51, 46-53	8.1
46	45	4.3
50	51, 51-55	4.0
51	50, 45-50, 48-50, 49-50, 52, 52-63	13.0
52	51, 53, 53-55	4.1
53	45-52, 48-52, 49-52, 50-52, 52	7.7

Clinical Trials: Antisense Oligonucleotides (AONs) and DMD

In 2013, clinical trials with two competitive SSO drugs were underway to treat DMD, the two separate SSO compounds, Eteplirsen (AVI-4658, initiated by AVI Biopharma, now Sarepta Therapeutics) and Drisapersen (PRO051, initiated by Prosensa/GlaxoSmithKlein), that intend to cause skipping of dystrophin exon 51[28,32,33]. These SSOs cover the same target sequence, differing in size (eteplirsen containing 10 more nucleotides) and in their chemical composition, as eteplirsen is based on phosphoramidate morpholino chemistry (PMO), and drisapersen is based on phosphorothioated 2'-O-methyl RNA chemistry (2OMe-PS)[28]. 2OMe-PS oligonucleotides comprise chemically synthesized, negatively charged, single stranded RNA molecules with a phosphorothioate (PS) backbone that hybridizes to the target exon, being this PS moiety very stable and resistant to intracellular endonucleases and exonucleases, and in

order to further increase the resistance to nuclease activity, in C2' the hydrogen is replaced by a methyl group[31]. Conversely PMO oligonucleotides are developed with replacements of the deoxyribose moiety with a morpholine ring and of the phosphodiester link by an uncharged phosphorodiamidate link[31]. Both drugs triggered exon 51 skipping and production of some dystrophin protein following intramuscular injections[33]. Despite its success in this first clinical trial, a phase III clinical trial with Drisapersen failed to meet the primary endpoint of a statistically significant improvement in the 6 Minute Walking Distance Test (6MWT) compared to placebo[33]. It was later identified a confounding variable present in the study - the age of the patients, patients with ages inferior to 7 years old performed better than the older patients (personal communication). The AONs in clinical trial are finishing phase III and currently awaiting approval from the Federal Drug Administration (FDA) and European Medicines Agency (EMA). A number of other SSOs (Prosensa, 2013) targeting different exons within the dystrophin gene are also in early clinical and preclinical development for skipping of exons 44, 45, 52, 53 and 55[28].

A new approach: LNA-modified oligonucleotides (LNA-AON)

Since the AONs already in clinical trials have shown limited success regarding clinical efficacy, we aimed to test if Locked Nucleic Acid (LNA)-modified oligonucleotides (LNA-AON) could be used for splicing modulation therapies with increased efficiency. LNAs are a general and versatile tool for specific high-affinity recognition of single-stranded DNA (ssDNA) and single-stranded RNA (ssRNA)[34]. In LNA-AONs, a varying number of natural nucleotides are replaced with nucleotide analogs carrying an altered sugar moiety, in which the ribose 2'-O- and 4'-C-atoms are connected via a methylene bridge[35]. LNA belongs to the so-called "third generation" of modified nucleotides, with improved properties in terms of increased duplex stability, high sensitivity, good mismatch discrimination, low toxicity and increased metabolic stability[35]. Several studies reported that no adverse effects of LNA-AONs on cell vitality have been observed at their biologically effective concentration or dosages[35]. Miravirsen is the first LNA-AON developed as a therapy. It antagonizes miR-122 in patients chronically infected with the hepatitis C virus (HCV), and was developed after observations that this virus could only replicate in the presence of miRNA-122, a liver specific microRNA that plays a pivotal role in regulating hepatic functions such as lipid metabolism and stress response. Current clinical trials suggest that this may be an effective and safe strategy for this patients[36]. Numerous studies demonstrate the enormous potential of LNA-AONs in basic and applied research, as well as in molecular medicine and therapeutics[35].

With this work we expect to show the applicability of short LNA-modified oligonucleotides for Duchenne Muscular Dystrophy gene therapy, paving the way for a search of more efficient methods for gene therapy splicing modulation in systemic treatment of an inherited genetic disease.

Materials and Methods

Myoblast Derived Cell Lines Culture and Differentiation

Myoblast derived cell lines from a patient (DM8036 cell line with a deletion of exons 48-50 in the *DMD* gene) and a control individual (KM155 cell line) were kindly provided by Vincent Mouly from the Institut de Myologie UPMC Université Paris 6, France (Mamchaoui et al. 2011)[37]. The myoblast derived cell lines were maintained with Skeletal Muscle Cell Growth Medium (PromoCell, Cat. No. C-23060), a medium optimized for expansion of human skeletal muscle cells that contains low-serum (5% v/v), at 37^{o}C with 5% carbon dioxide.

To induce differentiation, i.e. the fusion of myoblasts to myotubes with typical multinucleated syncythia, we monitored the cell density by microscopy and induced differentiation when cell confluence was approximately 80%, nearly 24h after seeding the cells at high density replacing the medium. Differentiation medium was DMEM (Gibco, Cat. No. 41966-029) containing ITS (Sigma, Cat. No. I3146-5ML), a general cell supplement, containing a mixture of recombinant human insulin, human transferrin, and sodium selenite. Cells were monitored by microscopy and after two days of differentiation if the presence of syncythia was observable, the cells were collected for RNA purification or protein extraction.

LNA-AON Structure and Transfections

From our group previous (unpublished) experiments, that compared different sequences and lengths of LNA-AONs, this LNA-AON was selected because it presented the greatest capability of inducing skipping of exon 51 in myoblast derived cell lines. It has the sequence 5'- AGGAA-GATGGCATTTC -3' (DNA LNA-AON) and contains a fully phosphorothioate modified backbone, presenting 60% LNA, with two LNA-modified nucleotides at the 3'-end and one LNA-modified nucleotide at the 5'-end. The 16mer LNA-AON purchased from Exiqon.

For experiments, DM8036 cells were seeded in a P24wells plate (TPP, Cat. No. TPP92024; RNA purification and immunocytochemistry) or in a P12wells plate (Corning, Cat. No. CORN3513; protein extraction) after adding the transfection reagent (Lipofectamine RNAimax, Invitrogen, Cat. No. 13778-075) and the LNA-AON, in the intended concentration, in each well. For a growth area of 1.9cm^2 (P24wells plate), 1μL of Lipofectamine RNAimax was used in 15μL of Optimem (Gibco, Cat. No. 31985-047). Different final concentrations of LNA-AONs were tested ranging from 5 to 500nM. DM8036 cells mock transfected with optimem were used as a negative control, and KM155 cells (dystrophin expressing control individual cell line) mock transfected with optimem were used as a positive control.

Animals

Animal procedures were performed in accordance with the guidelines of the European Community guidelines (Directive 2010/63/EU), Portuguese law on animal care (1005/92), and approved by the *Instituto de Medicina Molecular* Internal Committee and the Portuguese Animal Ethics Committee (*Direcção Geral de Veterinária*). Exon 52-deficient X chromosome-linked muscular dystrophy mouse model (*mdx52* mice) was kindly provided by Shin'ichi Takeda from the National Center of Neurology and Psychiatry, in Japan (Aoki et al. 2012)[38] and C57BL/6J mouse model, purchased from Charles River, were used in this study.

LNA Treatment of *mdx52* Mice

Mdx52 mice (n= 5/6 per group) were treated with five tail intravenous injections of 1mg/kg LNA-AON or saline solution (controls), in an approximate volume of 100μL each injection, at biweekly intervals (every 2 weeks). Treatment started when animals were 5-week old. One week after the last injection, functional and behavioral testing of the animals was performed using Grip Strength, Wire Hang, OpenField and RotaRod tests to assess the motor deficient phenotype in mice. The animals were examined 2 weeks after the final injection and euthanized via eutasil (CEVA) and cervical dislocation. Cardiac puncture was performed for terminal blood collection for analysis of specific biomarkers (creatine phosphokinase (CPK), blood urea nitrogen (BUN), creatinine, aspartate transaminase (AST) and alanine transaminase (ALT)). Muscles (Gastrocnemius - GC, Tibialis Anterior - TA, Heart - H and Diaphragm - D) were isolated immediately, snap frozen in liquid nitrogen and stored at -80ºC for immunohistochemistry, Western Blotting and reverse transcription PCR (RT-PCR).

Blood Analysis and Muscle Functional Testing

Blood, left at room temperature (RT) for 1h was centrifuged at 13500 rpm for 10min (Eppendorf) and the plasma was collected and sent to analysis of biochemical markers: creatine phosphokinase (CPK), blood urea nitrogen (BUN), creatinine, aspartate transaminase (AST) and alanine transaminase (ALT), by VetinLab (Veterinary Clinical Analysis, Lisbon).

The functional and behavioral testing of the animals was performed using different Grip Strength, Wire Hang, OpenField and RotaRod tests to assess motor deficient phenotype in mice[39,40], during the light period of the cycle, in a silent room, under dim light.

a. **Grip Strength** (SOP: DMD_M.2.2.001, SMA_M.2.1.002) – widely-used non-invasive method designed to evaluate mouse limb strength. We performed 3 assays per trial and 3 trials in total of the tests:

   - Wire Hang Test – the animals are placed in a wire grid, that is inverted, the duration of the test is 1min and the latency to fall is registed;
   - Automatic Grip Strength – the mouse grasps a horizontal metal bar or grid while being pulled by the tail. The bar or grid is attached to a force transducer (PCE

instruments, Cat.No. PCE-FM50) that provides the peak pull-force achieved.

b. **OpenField** – The simplest test of locomotor activity that involves observing and recording an animal's movements around an open-field arena. The OpenField protocol used was adapted from Coelho et al 2014[40]. The animals were placed in a designated corner of a square apparatus, surrounded by vertical walls (66cm x 66cm x 66cm) – open-field arena. They freely explored the maze for 5 min. Their movements were recorded and analyzed using the video-tracking software – SMART®. The reference point used by the software to determine the position of the animal was the center of the mouse's dorsum. Measurements of locomotor activity: the total distance traveled and the average speed were determined. At the end of the 5 min test, the animal was removed from the open-field arena and placed into its home cage.

c. **RotaRod** - used to assess sensorimotor coordination and motor learning in rodent models. The subjects are placed in a rotating rod and the latency to fall is recorded, an habituation period is needed.

Habituation Period (2-3 days)	Animals are placed on the rod at 8-12 rpm fixed rotation until they are able to stand unaided on the rod (3 trials per day separated by 30min each).
Test day	Fixed Rotation Protocol The animals are placed on a rod which accelerates to and then constantly rotates at the required velocity (12rpm). Accelerating Protocol The animals are placed in a rod that accelerates quickly from 0-4 rpm and then gradually from 4-40 rpm during a period of 5min. Attention: If animals fall before 7 rpm is reached they are placed back on the rod and it does not count as a fall. 3 trials are averaged to give the latency to fall of each animal.

A trial is complete when the animal falls or the time period ends. All the information regarding functional and behavioral testing of the mice was obtained from the websites http://www.treat-nmd.eu/research/preclinical/dmd-sops/ and http://sbfnl.stanford.edu/cs/bm/sm/.

RNA Isolation from Cell Extracts

Total RNA was extracted using PureZol (BioRad, Cat. No. 732-6890), according to the manufacturer's instructions. DNAse I treatment (Roche, Cat. No. 4716728001) and acidic phenol extraction with UltraPure™ Phenol:Cloroform:Isoamyl Alcohol (25:24:1, v/v; Invitrogen, Cat. No. 15593-031) were performed to additionally purify the RNA. Quantification and purity evaluation by A260/280 ratios were assessed using the spectrophotometer Nanodrop2000 (ThermoScientific)

RNA Isolation from Muscle Samples

Total RNA was obtained from 30mg fragments of frozen muscle. The Minibeadbeater (BioSpec Products) was used to disrupt and homogenize the tissue, using 1.0mm diameter zirconia-silica

beads (BioSpec Products), 3min. Total RNA was purified with RNeasy® Fibrous Tissue Mini Kit (Qiagen, Cat. No. 50974704), according to the manufacturer's instructions.

RT-PCR Protocol

The primer sequences for the PCR were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/), BLAST was performed in Ensembl (http://www.ensembl.org/index.html) and to check for self- and hetero-dimers OligoAnalizer 3.1 (http://eu.idtdna.com/calc/analyzer) was used. Primers are listed in Table 2.

Six hundred nanograms of RNA template was used for a 20μL retrotranscription reaction using Transcriptor High Fidelity cDNA Sinthesis Kit (Roche, Cat. No. 5081963001), according to the manufacturer's instructions at 55^{o}C for 90min, followed by 5min at 85^{o}C for degradation of the reverse transcriptase (BioRad MyCycler^{TM} Thermal Cycler). For a 20μL retrotranscription reaction, 1.2nmol of a specific primer (listed in Table 2) was used. The cDNA product was diluted 1/7.5 and 4μL were then used as the template for PCR in a 10μL reaction volume with KAPA2G^{TM} Fast Ready Mix (KapaBiosystems, Cat. No. KK5609). The cycling conditions were 95^{o}C for 5min, then 45 cycles: 95^{o}C for 15sec, 58^{o}C for 30sec, 72^{o}C for 15sec.

Table 2: Primer sequences used in this study.

Name	Species	Sequence	Amplicon Size
Specific Primer for RT			
mDMD_VP_e53R	Mouse	TCCTTAGCTTCCAGCCATTG	—
DMD_e54R	Human	GGAGAAGTTTCAGGGCCAAG	—
Forward Primer			
mDMD_VP_e50F	Mouse	GAGTGGGAGGCTGTAAACCAT	Skipped: 194bp
Reverse Primer			
mDMD_VP_e53R		TCCTTAGCTTCCAGCCATTG	Unskipped: 427bp
Forward Primer			
DMD_e47F	Human	ACCCGTGCTTGTAAGTGCTC	Skipped: 361bp
Reverse Primer			
DMD_e53R		TGACTCAAGCTTGGCTCTGG	Unskipped: 594bp

PCR products were separated on a 2% (wt/wt) agarose gel. The molecular weight marker 1Kb Plus DNA ladder (Invitrogen, Cat. No. 10787-018) was used. Digital images were obtained using the Chemidoc XRS+ system (BioRad) and analyzed using the Image Lab 5.2 software (BioRad).

Protein Extraction from Cell Extracts

The protein extraction protocol was adapted from Winter et al. 2012[41]. Cultured cells on P12wells plates were washed briefly with phosphate-buffered saline (PBS) at RT, and lysed and homogenized in 40μL treatment buffer (100mM Tris-HCl pH 6.8, 20% sodium dodecyl sulfatel (SDS)), in a surface area of 3.8cm^2 (P12wells plate) for 5min. Protein concentration was determined using the Pierce^{TM} BCA Protein Assay kit (ThermoScientific, Cat. No. 1513-7485), according to the manufacturer's instructions. This kit is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. Bovine Serum Albumin (BSA) is used as a protein standard for the determination of the protein concentration and the absorbance of the protein extracts is measured at 562nm. After protein quantification 1/100μL Benzonase (Sigma, Cat. No. E1014-25KU) and 0.5M $MgCl_2$ (final concentration of 14mM) was added, this endonuclease degrades all forms of DNA and RNA (single stranded, double stranded, linear and circular), even in the presence of SDS. After incubation for 15min at room temperature (RT), the homogenate was completed with 13.3μL of a 0.04% bromophenol blue, 20% dithiothreitol (DTT) and 80% glicerol solution to contain 75mM Tris-HCl pH 6.8, 15% sodium dodecyl sulfate, 5% dithiothreitol, 20% glycerol and 0.01% bromophenol blue and boiled at 98^{o}C for 5 min.

Protein Extraction from Muscle Samples

Muscles were homogenized using the treatment buffer previously described (100mM Tris-HCl pH 6.8, 20% sodium dodecyl sulfatel) and 1.0mm diameter zirconia-silica beads (BioSpec Products) in the Minibeadbeater (BioSpec Products), 3min. Protein concentration determination and completion of the protein extracts was performed as above. After 5min at 98^{o}C, the homogenate was then sonicated using an ultrasonic bath at 35kHz (VWR Ultrasonic Cleaner), and centrifuged (Eppendorf) at 20 000g, 4^{o}C for 30 minutes.

Western Blot Protocol

1-5μg of protein was loaded on a 7% polyacrylamide gel and run for 90min at RT: 10min at 60V + 80min at 100V (BioRad). Precision Plus Protein^{TM} Standard (BioRad, Cat. No. 161-0373) was used. Gels were blotted to nitrocellulose membranes (Whatman Protran BA 85 Nitrocellulose 0.45um 200x200mm, Cat. No. 10401191), in a Tank Transfer System (BioRad Mini Trans-Blot® Cell) with 300mA for 90min at RT (BioRad Power Pac Basic^{TM}). The membranes were stained with Ponceau S (Sigma, Cat. No. P3504-10G) to confirm the efficiency of the transference of the protein to the membrane. The membranes were marked and cutted to incubate separately the primary antibody from the loading control. Blots were blocked with 5% non-fat dried milk in Tris-buffered saline (TBS) plus 0.05% Tween-20 (TBST) followed by an overnight incubation at 4^{o}C with a rabbit polyclonal anti-dystrophin antibody (dilution 1/1000; Abcam, Cat. No. ab85302) in 5% non-fat dried milk in TBST. Goat Anti-Rabbit IgG (H+L)-HRP (dilution 1:3000; 60min at RT; BioRad, Cat. No. 1706515) was used as a secondary antibody.

The polyclonal antibody rabbit anti-Lamin A/C (dilution 1:10000; overnight at 4°C; H-110, Santa Cruz, Cat. No. sc-20681) was used as a loading control. Digital images were captured using the Chemidoc XRS+ system (BioRad) and analysed using the Image J software. To estimate the molecular weight of the truncated dystrophin protein the websites web.expasy.org/translate and www.bioinformatics.org/sms2/protein-mw.html were used.

Immunocytochemistry

For microscopy analysis, cells were transfected and seeded onto 0.1% gelatin coated glass coverslips (10x10mm^2) on P24wells plates. After 7 days differentiation, the cells were fixed with 3.7% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), 10min at RT, and permeabilized with 0.5%Triton x100 in PBS, 10min at RT. The cells were incubated for 30min at RT in a blocking solution containing 1%BSA and 0.05%Tween20 in PBS, all antibodies were diluted in this blocking solution. The cells were stained with a polyclonal rabbit anti-dystrophin antibody (dilution 1/100, 60min incubation at RT; Abcam, Cat. No. ab85302). Rhodamine (TRITC)-conjugated affinipure donkey anti-rabbit was used as a secondary antibody (dilution 1/200, 60min incubation at RT, Tetramethyll Rhodamine Isothiocyanate (TRITC); Jackson ImmunoResearch Laboratories Inc., Cat. No. 711-025-152). 0.1μg/mL 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei, 10min incubation at RT; Sigma, Cat. No. D9542-5MG). Vectashield Mount Medium (Vectorlabs, Cat. No. H-100) was used as a mounting medium, the borders of the coverslips were sealed with nail polish.

Immunohistochemistry

At least five 10μm cryosections were cut from the muscles of interest (tibialis anterior, gastrocnemius, diaphragm and heart) and fixed in ice cold acetone, 10min and air dried. Incubation with blocking solution, staining with antibodies anti-dystrophin and TRITC, and DAPI staining were performed as above.

Microscope Image Acquisition and Analysis

Digital images were captured using a Zeiss LSM 710 Confocal Point-Scanning Microscope, with 20x (immunohistochemistry) and 40x (immunocytochemistry) objectives using the lasers Diode 405-30 (405nm) and DPSS 561-10 (561nm). Digital images of maximum intensity projections, of 3 stacks spaced by 1μm, were analyzed with the software Image J and LSM 5 Image Browser (Zeiss).

Statistical Analysis

Statistical differences were assessed with a Mann-Whitney-Wilcoxon Test, using custom R (version 3.2.1) scripts. All data are reported as mean values $\pm$standard deviation (SD). The level of significance was set at $P < 0.05$. The software Graphpad Prism 6 was used for dose-response curves presentation and EC_{50} determination.

Results and Discussion

In vitro Evaluation of the Splicing Modulation

A myoblast derived cell line from a patient (DM8036 cell line)[37], that does not produce the dystrophin protein due to a deletion of exons 48-50 in the *DMD* gene, was transfected with a LNA-AON targeting exon 51, for *in vitro* induction of skipping of DMD-exon 51 and consequent restoration of protein production. A myoblast derived cell line from normal a individual (KM155 cell line)[37] was mock transfected and used as a positive control. Skipping of exon 51 allows for restoration of the reading frame of the dystrophin mRNA, correcting the frameshift and subsequently leading to the production of a truncated but partially functional dystrophin protein. From previous (unpublished) experiments, that compared different sequences and lengths of LNA-AONs, was selected a LNA-AON for this work because it presented the greatest capability of inducing skipping of exon 51 in myoblast derived cell lines. Different concentrations of the LNA-AON were tested, ranging from 5-500nM. In order to examine the exon 51 skipping at the transcript level and explore restoration of protein production, RT-PCR techniques and Western Blot and Immunocytochemistry techniques were employed.

Exon 51 Skipping at the Transcript Level

Myoblast derived cells (DM8036 patient cell line) were transfected with different concentrations of LNA-AON. Differentiation was induced 24h after transfection and extraction and purification of total RNA was performed after 2 days differentiation. Before harvesting the cells, differentiation was monitored by phase-contrast microscopy where multinucleated myotubes were observable. Retrotranscription was performed with a specific primer for DMD-exon 54 and the PCR protocol used was optimized do detect skipping of exon 51 in total mRNA, with only one round of amplification. In all the literature reviwed, amplification was done with nested PCR[42,43]. In our hands nested PCR also worked, but is not usefull for a semi-quantitative approach. Actualy Spitali et al 2010[44] compared different techniques for quantification of exon skipping levels in AON-treated *mdx* mouse muscle, and demonstrated that with a two-round amplification PCR (nested PCR), the skipping levels were generally overestimated. With our protocol, no skipped isoform bands were observed in the negative control, which consisted of the same cells mock transfected. The results obtained, with the RT-PCR, show effective skipping of exon 51 at the transcript level (Figure 5a and b) in dystrophic myoblasts transfected with the LNA-AON at concentrations as low as 5nM.

We also tryed the approach of qRT-PCR to quantify the skipping of exon 51 in total mRNA,

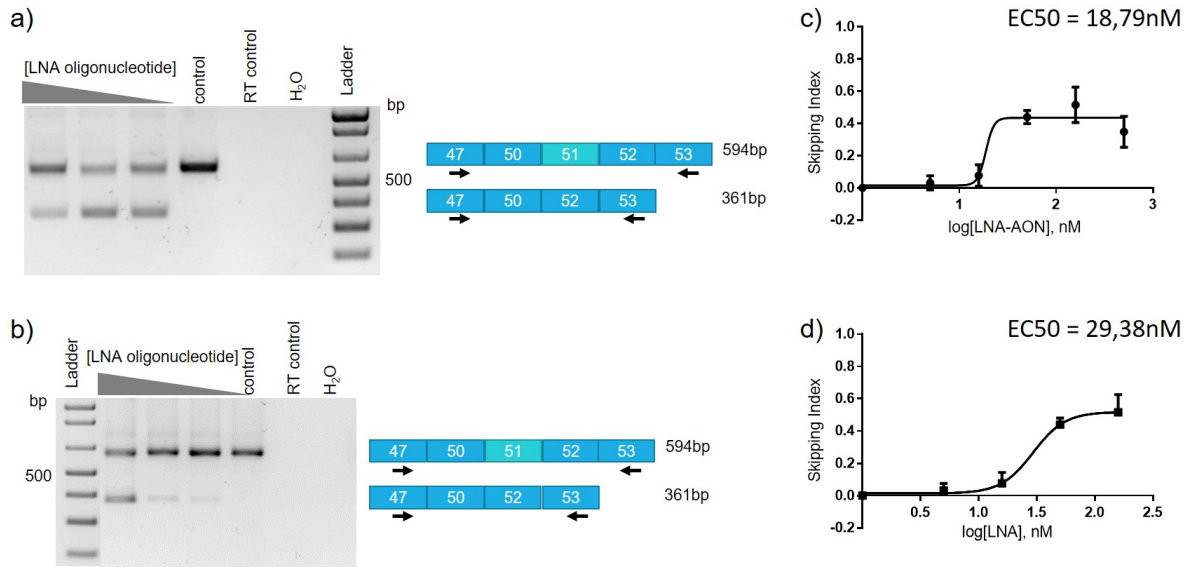

Figure 5: **Efficacy of exon 51 skipping in a myoblast derived cell line (DM8036 patient cell line)**. a) RT-PCR results after transfection of the LNA-AON with different concentrations (500, 158, 50nM and a negative control mock transfcted) and two days differentiation. b) RT-PCR results after transfection of the LNA-AON with different concentrations (50, 15.8, 5nM and a negative control mock transfected) and two days differentiation. c) and d) Dose-response curves obtained considering the skipping index and the LNA-AON concentration transfected. In d) the values for the 500nM were excluded from the calculations.

however construction of exon junction primers that could efficiently discriminate between the exon junctions of exons 47-51 (unskipped transcript) versus exons 47-52 (skipped transcript) proved difficult: we could obtain an amplification band with primers targeting the skipped isoform even in the negative control. Since the dystrophin mRNA is a low abundance transcript[45], detections of small variations of alternative spliced transcripts could be difficult at the RNA level if primers and the RT-PCR strategy are not optimized.

From the results obtained, a dose-response curve was originated using the software Graphpad Prism 6. The calculations were made considering the Skipping Index and the concentration of the LNA-AON (Figure 5c and d). The Skipping index was calculated accordingly with the equation: $SkippingIndex = exon51_{\text{exclusion}}/(exon51_{\text{exclusion}} + exon51_{\text{inclusion}})$. To calculate the Skipping Index, the relative quantification of the intensityof the skipped and unskipped bands in the agarose gel was assessed using the software ImageLab 5.2. From the dose-response curve obtained (Figure 5c), the calculation of the half maximal effective concentration (EC_{50}) was performed, this value refers to the concentration of a drug which induces a response halfway between the baseline and maximum, i.e. 50% of the maximum effect is observed. The EC_{50} value obtained was of 18,79nM. But since we can observe that the 500nM concentration presents a lower efficiency than the 158nM concentration, not presenting the maximum effect observed and that the program was not taking into account the decrease at the highest concentration tested, we excluded the 500nM concentration values and calculated a new dose-response curve (Figure 5d). From this new curve, we obtained a EC_{50} value of 29,38nM, which indeed does not differ greatly from the previously obtained. An explanation for this observation

might be a certain toxicity of this 500nM concentration for cell viability i.e. the preferential death of the cells with more uptake of the LNA-AON.

In order to examine if the effect of the LNA-AON is long lasting, the myoblast derived cells were transfected once with the different concentrations of the LNA-AON and were incubated in differentiation medium for two weeks. After two weeks differentiation, total RNA analysis with the optimized RT-PCR protocol, showed no detection of skipping of exon 51 (Figure 6). This experiment suggests that repeated administration of the treatment is necessary for a continued effect since this therapy is at the RNA level.

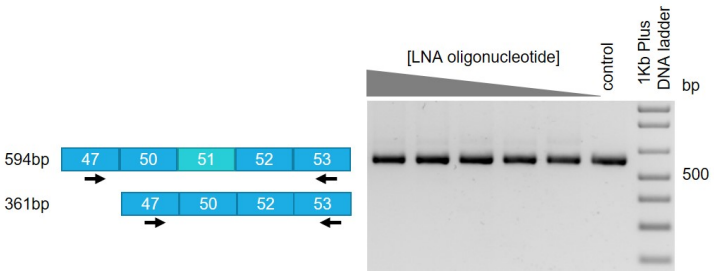

Figure 6: Exon 51 skipping is not detected after two weeks differentiation in myoblast derived cell lines (DM8036 patient cell line). The concentrations of transfected LNA-AON ranged from 5-500nM (500, 158, 50, 15.8, 5 and a negative control mock transfected).

From studies performed by Tennyson, we know that the human dystrophin gene with its 79 exons spanning over 2300kb requires approximatly 16h to be transcribed[46], and that the half-life of the dystrophin mRNA is 15.6 $\pm$2.8h in cultured human fetal myotubes[45]; this estimative was obtained using actinomycin D which has the ability to inhibit transcription, by binding DNA at the transcription initiation complex and preventing elongation of the RNA molecule by RNA polymerase. There are not considerable experimental evidences of AON turnover, it would be very important to develop this experiments for further improvement of activity and safety of AONs[47].

Restoration of Protein Production

Myoblast derived cells (DM8036 cell line) were transfected with different concentrations of LNA-AON. A myoblast derived cell line from normal individuals (KM155 cell line) was mock transfected and used as a positive control for dystrophin expression. Differentiation was induced 24h after transfection and for Western Blot, protein extracts were prepared after 2 days differentiation. Before harvesting the cells, differentiation was monitored by phase-contrast microscopy where multinucleated myotubes were observable. With the Western Blot protocol used, in the positive control, there was detection of only one band in the membrane, corresponding to the size expected for the control protein (427kDa) (Figure 7a), using 1μg of total protein extract loaded in the polyacrylamide gel. In the negative control, myoblast derived cells from patients (DM8036 cell line) mock transfected, there was no detection of the dystrophin protein. The estimated size for the truncated dystrophin protein is of approximately 400kDa, having in consideration the deletion of exons 48-50 and the skipping of exon 51. Restoration of dystrophin

protein production in dystrophic myoblasts transfected with LNA-AONs was detected (Figure 7b), with concentrations as low as 15.8nM of transfected LNA-AON.

From the results obtained, calculation of a dose-response curve was originated using the software Graphpad Prism 6. This calculations were made considering the dystrophin protein intensity of bands of the dystrophic myoblasts (DM8036 cell line) normalized to the positive control (KM155 cell line) and the concentration of the LNA-AON (Figure 7c and d). The relative quantification of the intensity of the bands in the membranes was assessed using the software ImageJ, the background was subtracted to the intensity of each band and each dystrophin level was obtained in relative quantification to the loading control (the lamin A band was chosen), for each sample, being afterwards normalized to the dystrophin relative quantification of the positive control. From the dose-response curve (Figure 7c), the EC_{50} value obtained was of 48,08nM. Since in the Western Blot results, the 500nM concentration presented a lower efficiency than the 158nM concentration (as we have seen already in the RT-PCR assay), we excluded the 500nM concentration values from the graphic and calculated a new dose-response curve (Figure 7d). From this new curve, we obtained a EC_{50} value of 49,43nM, that does not differ greatly from the previously obtained. It would be important to carry out an Western Blot experiment to see if after two weeks differentiation there is still detection of dystrophin protein restoration in dystrophic myoblasts (DM8036 cell line) transfected with the different concentrations of LNA-AON, but that question could be even better addressed by a microscopy approach.

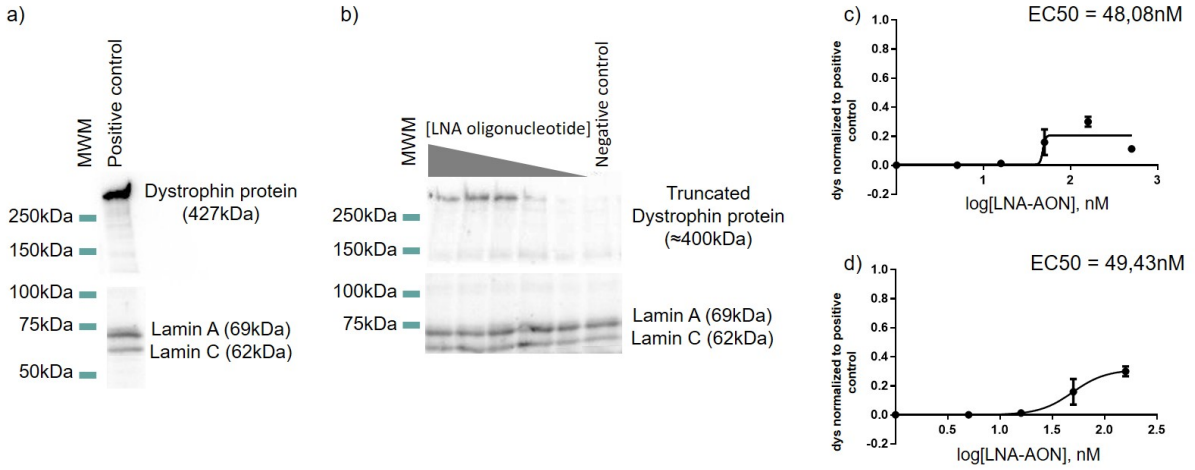

Figure 7: **Restoration of protein production after exon 51 skipping in a myoblast derived cell line (DM8036 patient cell line)**. a) Western blotting analysis of the KM155 normal individual cell line, used as a positive control. b) Western Blotting analysis of the different concentrations of LNA-AON transfected in the DM8036 patient cell line. c) and d) Dose-response curves obtained considering the intensity of the dystrophin bands relative quantites to the loading control, normalized to the positive control and the LNA-AON concentration transfected. In c) the values for the 500nM were excluded from the calculations.

By microscopy of immunolabeled cells we can address, beside the presence of dystrophin protein, its correct localization in the plasma membrane of differentiated cells. In the Immunocytochemistry protocol performed, after transfection with the different concentrations of the LNA-AON, induction of differentiation 24h after the transfection, fixation of the cells occurred

7 days after differentiation. In order to perceive if the cells were differentiated, i.e. if fusion of myoblasts had originated multinucleated myotubes, DAPI was used to stain the nuclei. Using myoblast derived cells from control individuals mock transfected (KM155 cell line - positive control), we could ascertain if the antibody used was detecting the dystrophin protein and where it was localized. In the positive control, dystrophin positive fibers are observed and the localization of the staining is in the periphery of the multinucleated cells. In the negative control, dystrophic myoblasts from patients (DM8036 cell line) mock transfected, there was no detection of dystrophin. Detection of dystrophin positive fibers in dystrophic myoblasts (DM8036 cell line) transfected with LNA-AONs was detected, via Immunocytochemistry (Figure 8), with concentrations as low as 50nM. From all the experiments performed, the concentration that presented a higher efficiency of DMD-exon 51 skipping and dystrophin protein restoration was 158nM LNA-AON.We can see that the presence of dystrophin in treated cells is not as high as in control cells and that it does not occur in all the cells in the coverslip, but the localization in the plasma membrane is as expected, which makes these seem promising results.

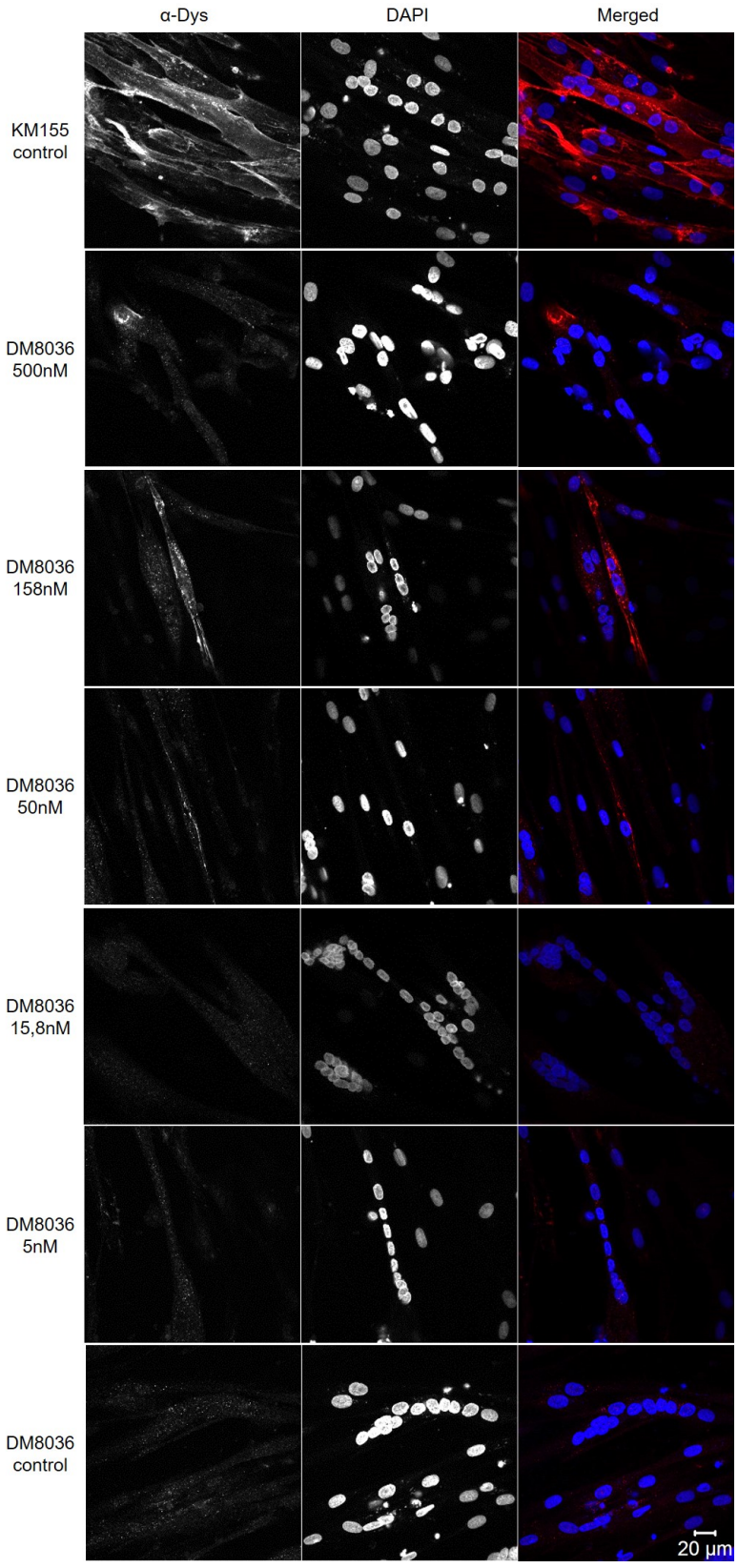

Figure 8: **Restoration of protein production after exon 51 skipping in myoblast derived cell lines (DM8036 patient cell line)**. Fluorescence immunocytochemistry analysis of the different concentrations of LNA-AON transfected in the DM8036 patient cell line, KM155 control individual cell line was used as a positive control. Polyclonal antibody ab85302 was used to detect dystrophin, DAPI was used to stain the nuclei.

In vivo Evaluation of the Splicing Modulation in *mdx52* mouse model

The *mdx52* mouse model, that presents a deletion of DMD-exon 52[38], was used for *in vivo* studies of splicing modulation by a LNA-AON targeting skipping of DMD-exon 51, to restore the reading frame of the dystrophin mRNA and subsequently lead to production of a truncated but partially functional dystrophin protein. *Mdx52* mice (n= 5-6 per group) were treated with five tail intravenous injections of 1mg/kg LNA-AON or saline solution (control) at biweekly intervals (every 2 weeks). One week after the final injection, functional and behavioral testing of the mice was performed to assess motor phenotype (Grip Strength, Wire Hang and OpenField). The mice were euthanized two weeks after the final injection and terminal blood was collected for analysis of specific biomarkers: creatine phosphokinase (CPK), blood urea nitrogen (BUN), creatinine, aspartate transaminase (AST) and alanine transaminase (ALT). Muscles (Gastrocnemius - GC, Tibialis Anterior - TA, Heart - H and Diaphragm - D) were isolated, snap frozen and stored for Immunohistochemistry, Western Blotting and RT-PCR analysis.

Blood Analysis and Muscle Functional Testing

In order to examine the functional phenotype, a battery of physiological and blood tests were performed after the five biweekly intravenous injections with the LNA-AON (Figure 9). High levels of serum creatine phosphokinase (CPK), an important enzyme in heart, brain and skeletal muscle, are present in muscular dystrophies, such as DMD, due to damage of the muscle tissue leading to leakage of CPK into the blood. In normal situations, low levels of CPK are present in the blood (see Supplemental Figure14b). If a reduction of the CPK levels was to be present, this would suggest the protection of muscle fibers against degeneration. To further monitor any potential toxicities in the major organs induced by the treatment with the LNA-AON, we compared a series of standard serum markers as indicators of liver and kidney dysfunction in treated and untreated *mdx52* mice (Figure 9a). Creatinine and blood urea nitrogen (BUN) are indicators of renal health, and aspartate transaminase (AST) and alanine transaminase (ALT) are measured clinically as biomarkers of liver health. AST is also used as a biomarker of muscle damage. No significant differences were detected between untreated and treated *mdx52* mice groups in the levels of AST, ALT, BUN and creatinine (Figure 9a). These data do not allude towards a toxic effect of the LNA-AON tested *in vivo*. We also could not find a significant reduction of CPK in treated mice, but there was a problem with getting the results from all the animals in this experiment.

In this study, this reduction was not observed when comparing treated and untreated *mdx52* mice (Figure 9a). A significant difference ($p = 0.01732$, Mann-Whitney-Wilcoxon Test) between the treated and untreated *mdx52* mice was observable in the OpenField test, the average velocity of the treated *mdx52* mice is greater than the untreated *mdx52* mice, but no significant improvement was observed in forelimb grip strength in treated *mdx52* mice compared with untreated *mdx52* mice (Figure 9b).

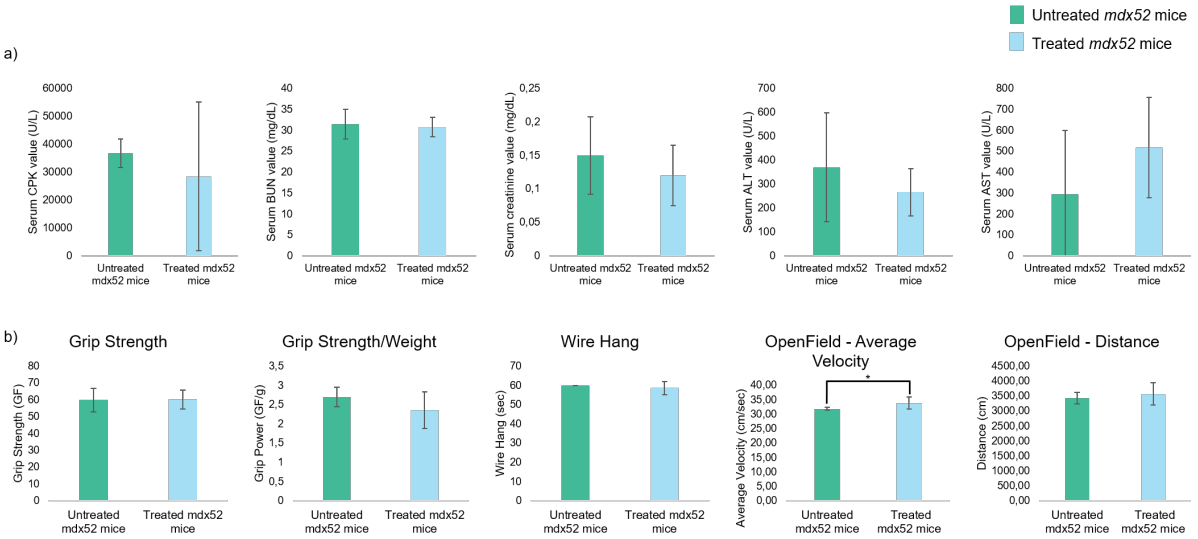

Figure 9: **No amelioration of the skeletal muscle function was observable in the *mdx52* mice**. a) Measurement of biochemical markers [creatine phosphokinase - CPK (U/L), blood urea nitrogen - BUN (mg/dL), creatinine (mg/dL), aspartate transaminase - AST (U/L) and alanine transaminase - ALT (U/L)] levels. b) Functional and behavioral testing of the mice was performed using Grip Strength (GF and GF/g), Wire Hang (sec) and OpenField (cm/sec and sec), in treated and untreated *mdx52* mice. Data (treated n=6, untreated n=5) are presented as mean $\pm$SD.

Exon 51 Skipping at the Transcript Level

Total RNA from muscles was extracted with an RNeasy® Fibrous Tissue Mini Kit, from approximately 30mg of tissue. Retrotranscription was performed with a specific primer for DMD-exon 53 and the PCR protocol used was the optimized protocol described in the section "Evaluation of the Splicing Modulation".

The negative controls (not shown) presented no band. A positive control was not used because we did not have one. As showed in Figure 10a, we did not detect any band corresponding to the skipped exon 51 isoform. This result can be interpreted by comparison with the result from Figure 6, because detection of skipping of exon 51 was not observable at the RNA level after two weeks differentiation in myoblast derived cell lines transfected once with the LNA-AON, it is comprehensible that detection of skipping of exon 51 at the RNA level in the *mdx52* mice was not observable, since the acquisition of the samples for RT-PCR analysis was made two weeks after the last injection with the LNA-AON.

Restoration of Protein Production

Transverse cryosections of the different muscles collected were performed for immunohistochemical staining of dystrophin in treated and untreated *mdx52* mice. C57BL/6J mice were used as a positive control. Dystrophin-positive clusters of fibers were detected in tibialis anterior and cardiac muscles of treated animals as exemplified in Figures 11 and 12), via immunohistochemistry. One could think that this dystrophin-positive fibers were likely to be naturally occurring revertant fibers, i.e. occasional isolated fibers that appear to express correctly lo-

calized dystrophin, as described[48,49,50] and also by our own observations on control animal muscles. Indeed revertant fibers were sporadically detected in the untreate *mdx52* mice mock injected, nevertheless the observations made in the transverse cryosections of the muscles of treated *mdx52* mice correspond to clusters of dystrophin-positive fibers in discrete regions of the muscle. Which leads to presuming that this fibers are indeed dystrophin-positive fibers therapeutically induced by the LNA-AON and not revertant fibers.

Despite being able to detect some dystrophin-positive fibers via Immunohistochemistry in the different muscles analyzed, dystrophin protein via Western Blot on treated animals was not detected as exemplified by Figure 10b. Protein extracts from the muscles were obtained from approximately 50mg of tissue. 5μg of protein were loaded in a polyacrylamide gel for Western Blot analysis with the optimized protocol. There is the possibility that not enough dystrophin protein is present in the treated protein muscle extracts, to allow detection via Western Blot. It is also important to note that protein turnover is elevated in muscle of *mdx* mice, rates of muscle protein synthesis and degradation are higher in *mdx* mice than wt mice[51].

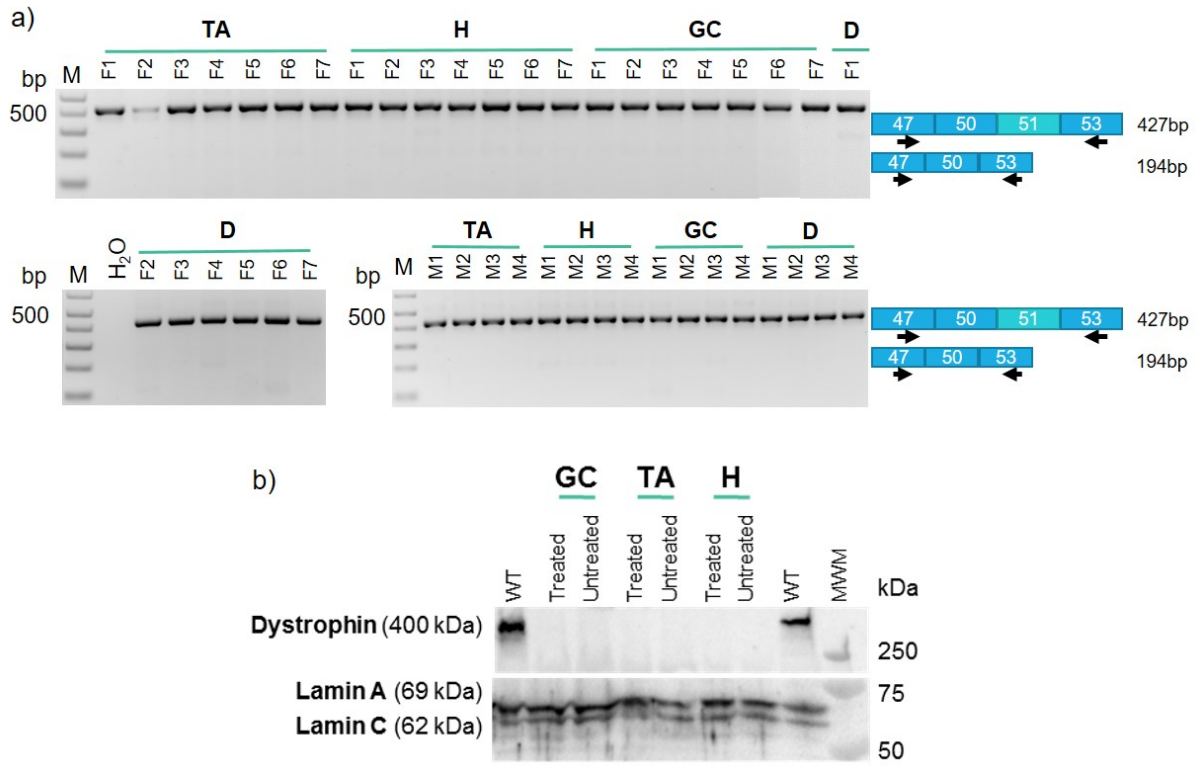

Figure 10: **Efficacy of exon 51 skipping in treated *mdx52* mice**. a) RT-PCR results to detect exon 51 skipping in various muscles (Tibialis Anterior - TA, Heart – H, Gastrocnemius - GC, and Diaphragm – D). M, molecular marker (1Kb Plus DNA ladder). b) Western blotting analysis to detect the expression of dystrophin in various muscles (TA, H, GC and D). Protein extracts from GC of C57BL/6J were used as a positive control. Treated animals: F1, F2, F5, F6, M2 and M3 and untreated mock injected animals: F3, F4, F7, M1 and M4 mice.

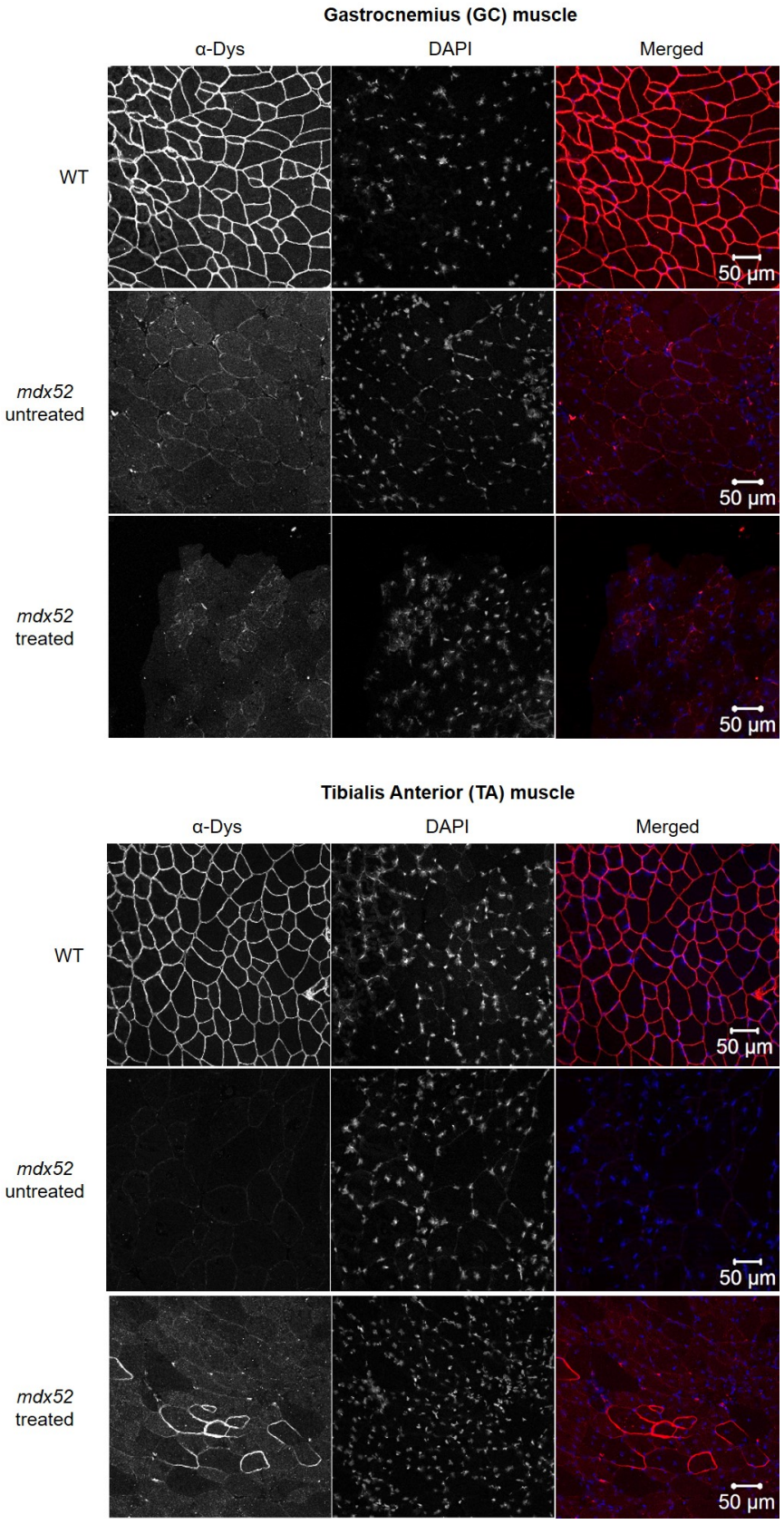

Figure 11: **Restoration of protein production after systemic injections in *mdx52* mice**. Fluorescence immunohistochemical staining of dystrophin in transverse cryosections of tibialis anterior (TA) and gastrocnemius (GC) muscles of treated and untreated *mdx52* mice and C57BL/6J mice, used as a positive control. Representative data are shown.

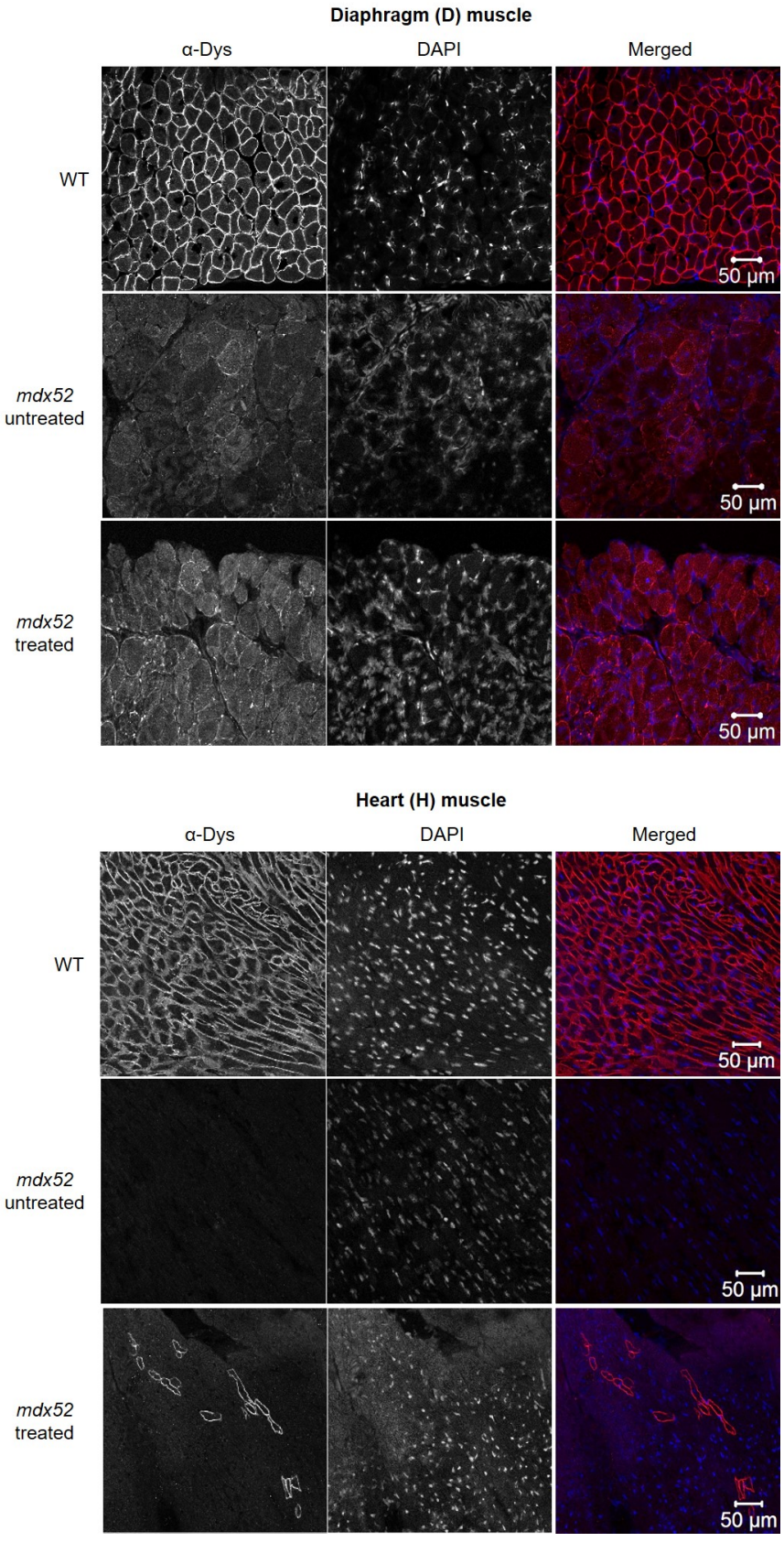

Figure 12: **Restoration of protein production after systemic injections in *mdx52* mice**. Fluorescence immunohistochemical staining of dystrophin in transverse cryosections of diaphragm (D) and heart (H) muscles of treated and untreated *mdx52* mice and C57BL/6J mice, used as a positive control. Representative data are shown.

In we compare in another experiment a LNA-AON with no single mismatch with the one we used in this work, the results wll be a good indicator of the absence/presence of off-target effects, and test if 100% homology is required for the LNA-AON to hibridize effectively and be able to allow splicing modulation with the purpose of restoring dystrophin protein production.

In this experiment we were able to see some effect of the injection of a LNA-AON towards reversion of the phenotype of dystrophin-negative muscular fibers in the DMD mouse model *mdx52*, but this effect was very low comparing with the already described by others[38]. A number of factors could have contributed to this result. 1) There is a single mismatch in the sequence of the LNA-AON when compared with the *DMD* gene sequence in mouse, that is not present on the human genome. 2) We were using a less concentrated solution of LNA-AON comparing with the literature[38]. 3) 5 week old animals are very small and the injection could have been not so effective. We performed only one experiment with a small number of animals.

Final Remarks

The aim of this project was to test if LNA-AONs could be used for splicing modulation therapies with increased efficiency, regarding the already studied different AON chemistries. Rescue of dystrophin expression by skipping exon 51 with an LNA-AON was shown in myoblast derived cell lines *in vitro*, and in dystrophic *mdx52* mice *in vivo* however with different efficiencies. It is noteworthy to mention that a recent study underscores the potential of exploring additional modifications (tricyclo-DNA AON chemistry) than the already studied[52].

Aartsma-Rus et al 2004[43] performed a comparative analysis of different AON chemistries for targeted DMD-exon 46 skipping in primary human myoblasts from a DMD patient, carrying a deletion of exon 45. A comparison of different chemistries was performed where 2OMe-PS (2'-O-methyl phophorothioate), morpholino (morpholino-phosphorodiamidate DNA), LNA (locked nucleic acid) and PNA (peptide nucleic acid) backbones were tested[43]. With that study, assessment of the efficiency of the different AONs was performed and observed that LNA induced higher skipping levels, however while inducing higher skipping efficiency it showed less sequence specificity. The conclusion of the study regarded that LNAs had a limitation, the increased risk of adverse effects elsewhere in the human genome, and considered the 2OMe-PS AONs the most favorable compound for targeted DMD-exon 46 skipping[43].

Since 2014 significant improvements in oligochemistry have been achieved. In this study, we characterized the rescue of dystrophin in patient cells using an LNA-AON which had already been shown in the group (data not shown) as the best of a set of LNA-AONs tested. The targeted skipping of DMD-exon 51 with the LNA-AONs did not induce skipping of adjacent exons, which was observable in the study performed by Aartsma-Rus et al 2004. The concentrations tested in the study performed in 2004[43] ranged from 100-500nM. In this study, the concentrations ranged from 5-500nM. We were able to detect skipping with a concentration 10 times lower than the one used in studies following the one performed in 2004 (500nM)**?** . We achieved skipping of exon 51, detected by RT-PCR, with an EC_{50} of 30nM and restoration of dystrophin protein production with an EC_{50} of 50nM. This values present the same order

of magnitude, which suggests that the results are robust. In all the different experiments performed *in vitro*, we could observe that the transfection with 500nM concentration presented a lower efficiency of skipping and a lower protein production than a lower concentration. An explanation for this observation might be a certain toxicity of this 500nM concentration for cell viability i.e. the preferential death of the cells with more uptake of the LNA-AON. The concentration that presented the best efficiency, in our hands, was 158nM. More studies should be performed, since the presented results are still preliminary, in order to exactly determine the EC_{50} value of this LNA-AON. Nonetheless the presented results, obtained with the cell model, seem very promising in order to achieve a good recovery of dystrophin protein in the myoblast cell line from DMD patients. Studies should also be performed to ascertain if the oligonucleotide is being degraded and calculate its half-life, since prolonged administration of the treatment is necessary for a continued effect because the therapy is at the RNA level.

In the future, free-uptake experiments should be performed with a range of concentrations to determine the EC_{50}, and to see how the LNA-AON designed performs without the assistance of lipofectamine for transfection. This assays, will show the efficiency of the oligonucleotide in entering the cell, and will allow us to understand if this oligonucleotide is the most suitable for *in vivo* experiments. In order to understand if this oligonucleotide is appropriate to be used in a mouse model, with the same efficiency obtained in the human myoblast derived cell lines, it is important that this experiments would also be performed in mouse myoblast derived cell lines.

In order to perform the *in vivo* evaluation of the splicing modulation, we based this experiments in a protocol performed by Aoki et al 2012[38]. In this protocol, it was performed bodywide skipping of exons 45-55, that cover the main mutational “hotspot” of the *DMD* gene, in *mdx52* mice by systemic antisense delivery of a mixture of ten AONs with the chemistry PMO, and this protocol proved feasible. In the experiments performed in this study, a single LNA-AON was systemically injected in the *mdx52* mice with the intent of inducing skipping of exon 51, in order to test the ability of rescuing dystrophin protein production and improvement of the phenotype caused by this disease in the animal model.

Intramuscular injections into a single site or into several sites of one muscle, could have been performed for measuring local effects of the LNA-AON, however it is difficult to envision global muscle targeting using this strategy[19]. For whole-body targeting of the musculature, a systemic delivery approach is required, relying the most current approaches on oral or intravascular delivery, the last being required for muscle targeting[19].

Preliminary studies (data not shown) where 10mg/kg injections were performed once in *mdx52* mice showed dystrophin protein restoration in TA and heart, however increased inflammation was also observable by histology. For this reason, the systemic and repeated delivery performed in this study was performed with injections of 1mg/kg LNA-AON. It would be interesting to perform a histological and immunohistochemical study (with specific markers) to see if this protocol reduced or not the inflammation process in the *mdx52* mice. The results obtained show absence of DMD-exon 51 skipping detection by RT-PCR, which is consistent with the results obtained *in vitro* with two weeks differentiation. The results obtained with the immunohistochemistry show the appearance of clusters of dystrophin positive fibers therapeutically

induced by the LNA-AON, without apparent increase in toxicity. Investigation of the concentration of AON present in the tissue after the treatment was not possible in this study, but an assay for measuring the AON concentration in tissue samples, based on a hybridization-ligation assay[53], has been developed for biodistribution analysis of AON uptake by skeletal muscles, heart, diaphragm, liver, spleen and kidney[41], and is being performed by Prosensa, a biotechnology company. We were able to detect dystrophin-positive fibers, however the efficiency of skipping was low. To increase the efficiency of skipping in *mdx52* mice, optimization of the sequence of the LNA-AON to test and of the delivery system of the oligonucleotides to the muscle is required, since whole-body treatment is challenging because the tissues involved are post-mitotic and 30 to 40% of the human body consists of muscle[10]. Standardization of the number of injections and weeks of treatment is required since they differ in the different chemistries studied, not allowing for equally comparison between the different splice-switching oligonucleotides (SSOs) being studied. Studies trying to optimize the delivery of AONs to certain tissues are underway, using AONs encapsulated in nanoparticles[54] or different chemical modifications[55]. It is important to understand the uptake mechanisms of the AONs *in vivo*, to improve delivery methodologies and increase the efficiency of the treatment. Improvement of exon-skipping efficiency and biodistribution of antisense oligonucleotides (AONs) may reduce the therapeutic dose and interval of administrations, minimizing the potential toxicity, off-target effects, and the cost burden, since the low abundance of the dystrophin mRNA transcript and its approximately 16h half-life as well as the higher protein turnover lead to the requirement of a lifelong repeated AON treatment[31,41].

Bibliography

[1] Liutao Du and Richard A. Gatti. Progress toward therapy with antisense-mediated splicing modulation. *Current Opinion Molecular Therapy*, 11(2):116–123, 2009.

[2] Jocelyn E. Krebs, Elliott S. Goldstein, and Stephen T. Kilpatrick. *Lewin's GENES XI*. 2014.

[3] Luca Cartegni, Shern L Chew, and Adrian R Krainer. Listening to silence and understanding nonsense: exonic mutations that affect splicing. *Nature reviews. Genetics*, 3(4):285–298, 2002.

[4] Annemieke Aartsma-Rus. Antisense-mediated modulation of splicing: therapeutic implications for Duchenne muscular dystrophy. *RNA biology*, 7(August):453–461, 2010.

[5] M. L. Hastings and a. R. Krainer. Pre-mRNA splicing in the new millennium. *Current Opinion in Cell Biology*, 13(3):302–309, 2001.

[6] R. Kole, R. R. Shukla, and S. Akhtar. Pre-mRNA splicing as a target for antisense oligonucleotides. *Advanced Drug Delivery Reviews*, 6(3):271–286, 1991.

[7] M. Soller. Pre-messenger RNA processing and its regulation: A genomic perspective. *Cellular and Molecular Life Sciences*, 63(7-8):796–819, 2006.

[8] a Javier Lopez. ALTERNATIVE SPLICING OF PRE-mRNA: Developmental Consequences and Mechanisms of Regulation. pages 279–305, 1998.

[9] Michael Krawczak, Jochen Reiss, and David N. Cooper. The mutational spectrum of single base-pair substitutions in mRNA splice junctions of human genes: Causes and consequences. *Human Genetics*, 90(1-2):41–54, 1992.

[10] Annemieke Aartsma-Rus and Gert-Jan B van Ommen. Progress in therapeutic antisense applications for neuromuscular disorders. *European journal of human genetics : EJHG*, 18(2):146–153, 2010.

[11] Annemieke Aartsma-Rus and Gert-Jan B van Ommen. Antisense-mediated exon skipping: a versatile tool with therapeutic and research applications. *RNA (New York, N.Y.)*, 13(10):1609–1624, 2007.

[12] Pietro Spitali and Annemieke Aartsma-Rus. Splice modulating therapies for human disease. *Cell*, 148(6):1085–1088, 2012.

[13] Kevin M. Flanigan. Duchenne and becker muscular dystrophies. *Neurologic Clinics*, 32(3):671–688, 2014.

[14] Guillaume-Benjamin-Amand Duchenne. The pathology of paralysis with muscular degeneration (Paralysie Myosclerotique), or paralysis with apparent hypertrophy. *British Medical Journal*, 2:541–542, 1867.

[15] Eric P. Hoffman, Robert H Brown, and Louis M. Kunkel. Dystrophin: The protein product of the Duchenne muscular dystrophy locus. *Cell*, 51:919–928, 1987.

[16] M Koenig, a H Beggs, M Moyer, S Scherpf, K Heindrich, T Bettecken, G Meng, C R Müller, M Lindlöf, and H Kaariainen. The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. *American journal of human genetics*, 45(4):498–506, 1989.

[17] a P Monaco, C J Bertelson, S Liechti-Gallati, H Moser, and L M Kunkel. An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. *Genomics*, 2(1):90–95, 1988.

[18] J S Chamberlain. Dystrophin levels required for genetic correction of Duchenne muscular dystrophy. *Basic and Applied Myology*, 7(3-4):251–256, 1997.

[19] Jane T Seto, Niclas E Bengtsson, and Jeffrey S Chamberlain. Therapy of Genetic Disorders-Novel Therapies for Duchenne Muscular Dystrophy. *Current pediatrics reports*, 2(2):102–112, June 2014.

[20] Marcella Neri, Silvia Torelli, Sue Brown, Isabella Ugo, Patrizia Sabatelli, Luciano Merlini, Pietro Spitali, Paola Rimessi, Francesca Gualandi, Caroline Sewry, Alessandra Ferlini, and Francesco Muntoni. Dystrophin levels as low as 30% are sufficient to avoid muscular dystrophy in the human. *Neuromuscular Disorders*, 17(11-12):913–918, 2007.

[21] Jeffrey Ehmsen, Ellen Poon, and Kay Davies. The dystrophin- complex The Dystrophin-Associated Protein Comple ex. *Journal of cell science*, 2002(115):2801–2803, 2002.

[22] Kristen J. Nowak and Kay E. Davies. Duchenne muscular dystrophy and dystrophin: pathogenesis and opportunities for treatment. *EMBO reports*, 5(9):872–876, 2004.

[23] Judith C T van Deutekom and Gert-Jan B van Ommen. Advances in Duchenne muscular dystrophy gene therapy. *Nature reviews. Genetics*, 4(10):774–783, 2003.

[24] Ryszard Kole, Tiffany Williams, and Lisa Cohen. RNA modulation, repair and remodeling by splice switching oligonucleotides. *Acta Biochimica Polonica*, 51(2):373–378, 2004.

[25] Burcu Bestas, Graham McClorey, Ulf Tedebark, Pedro M D Moreno, Thomas C Roberts, Suzan M Hammond, C I Edvard Smith, Matthew J a Wood, and Samir El Andaloussi. Design and application of bispecific splice-switching oligonucleotides. *Nucleic acid therapeutics*, 24(1):13–24, 2014.

[26] Joshua Lee and Toshifumi Yokota. Antisense Therapy in Neurology. *Journal of Personalized Medicine*, 3(3):144–176, 2013.

[27] Holly a. Kuzmiak and Lynne E. Maquat. Applying nonsense-mediated mRNA decay research to the clinic: progress and challenges. *Trends in Molecular Medicine*, 12(7):306–316, 2006.

[28] Petra Disterer, Adrianna Kryczka, Yuqi Liu, Yusef E Badi, Jessie J Wong, James S Owen, and Bernard Khoo. Development of therapeutic splice-switching oligonucleotides. *Human gene therapy*, 25(July):587–98, 2014.

[29] Z Dominski and Ryszard Kole. Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. *Proceedings of the National Academy of Sciences of the United States of America*, 90:8673–8677, 1993.

[30] Annemieke Aartsma-Rus, Ivo Fokkema, Jan Verschuuren, Ieke Ginjaar, Judith Van Deutekom, Gert J. Van Ommen, and Johan T. Den Dunnen. Theoretic applicability of antisense-mediated exon skipping for Duchenne muscular dystrophy mutations. *Human Mutation*, 30(3):293–299, 2009.

[31] Taeyoung Koo and Matthew J Wood. Clinical trials using antisense oligonucleotides in duchenne muscular dystrophy. *Human gene therapy*, 24(May):479–88, 2013.

[32] V Arechavala-Gomeza, K Anthony, J Morgan, and F Muntoni. Antisense Oligonucleotide-Mediated Exon Skipping for Duchenne Muscular Dystrophy: Progress and Challenges. *Current Gene Therapy*, 12:152–160, 2012.

[33] Qi-Long Lu, Sebahattin Cirak, and Terence Partridge. What Can We Learn From Clinical Trials of Exon Skipping for DMD? *Molecular therapy. Nucleic acids*, 3:e152, 2014.

[34] Shantanu Karkare and Deepak Bhatnagar. Promising nucleic acid analogs and mimics: Characteristic features and applications of PNA, LNA, and morpholino. *Applied Microbiology and Biotechnology*, 71(5):575–586, 2006.

[35] Arnold Grunweller and Roland K Hartmann. Locked Nucleic Acid Oligonucleotides: The Next Generation of Antisense Agents? *BioDrugs*, 21(4):235–243, 2007.

[36] Jihae Baek, Soowon Kang, and Hyeyoung Min. MicroRNA-targeting therapeutics for hepatitis C. *Archives of pharmacal research*, 37(3):299–305, March 2014.

[37] K Mamchaoui, C Trollet, A Bigot, E Negroni, S Chaouch, A Wolff, Pk Kandalla, S Marie, J Di Santo, Jl St Guily, F Muntoni, J Kim, S Philippi, S Spuler, N Levy, Sc Blumen, T Voit, We Wright, A Aamiri, G Butler-Browne, and V Mouly. Immortalized pathological human myoblasts: towards a universal tool for the study of neuromuscular disorders. *Skeletal Muscle*, 1(1):34, 2011.

[38] Yoshitsugu Aoki, Toshifumi Yokota, Tetsuya Nagata, Akinori Nakamura, Jun Tanihata, Takashi Saito, Stephanie M R Duguez, Kanneboyina Nagaraju, Eric P Hoffman, Terence Partridge, and Shin'ichi Takeda. Bodywide skipping of exons 45-55 in dystrophic mdx52 mice by systemic antisense delivery. *Proceedings of the National Academy of Sciences of the United States of America*, 109(34):13763–8, August 2012.

[39] Simon P Brooks and Stephen B Dunnett. Tests to assess motor phenotype in mice: a user's guide. *Nature reviews. Neuroscience*, 10(7):519–529, 2009.

[40] Joana E. Coelho, Pedro Alves, Paula M. Canas, Jorge S. Valadas, Tatiana Shmidt, Vania L. Batalha, Diana G. Ferreira, Joaquim a. Ribeiro, Michael Bader, Rodrigo a. Cunha, Frederico Simoes do Couto, and Luisa V. Lopes. Overexpression of Adenosine A2A Receptors in Rats: Effects on Depression, Locomotion, and Anxiety. *Frontiers in Psychiatry*, 5(June):1–8, 2014.

[41] Christa L Tanganyika-de Winter, Hans Heemskerk, Tatyana G Karnaoukh, Maaike van Putten, Sjef J de Kimpe, Judith van Deutekom, and Annemieke Aartsma-Rus. Long-term Exon Skipping Studies With 2'-O-Methyl Phosphorothioate Antisense Oligonucleotides in Dystrophic Mouse Models. *Molecular Therapy Nucleic Acids Nucleic Acids*, 1(9):e43, 2012.

[42] a. Aartsma-Rus. Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. *Human Molecular Genetics*, 12(8):907–914, April 2003.

[43] a Aartsma-Rus, W E Kaman, M Bremmer-Bout, a a M Janson, J T den Dunnen, G-J B van Ommen, and J C T van Deutekom. Comparative analysis of antisense oligonucleotide analogs for targeted DMD exon 46 skipping in muscle cells. *Gene therapy*, 11(18):1391–1398, 2004.

[44] Pietro Spitali, Hans Heemskerk, Rolf H a M Vossen, Alessandra Ferlini, Johan T den Dunnen, Peter a C 't Hoen, and Annemieke Aartsma-Rus. Accurate quantification of dystrophin mRNA and exon skipping levels in duchenne muscular dystrophy. *Laboratory investigation; a journal of technical methods and pathology*, 90(March):1396–1402, 2010.

[45] C N Tennyson, Q Shi, and R G Worton. Stability of the human dystrophin transcript in muscle. *Nucleic acids research*, 24(15):3059–3064, 1996.

[46] C N Tennyson, H J Klamut, and R G Worton. The human dystrophin gene requires 16 hours to be transcribed and is cotranscriptionally spliced. *Nature Genetics*, 9:184–190, May 1995.

[47] Tsuyoshi Yamamoto, Naoko Fujii, Hidenori Yasuhara, Shunsuke Wada, Fumito Wada, Naoya Shigesada, Mariko Harada-Shiba, and Satoshi Obika. Evaluation of Multiple-Turnover Capability of Locked Nucleic Acid Antisense Oligonucleotides in Cell-Free RNase H-Mediated Antisense Reaction and in Mice. *Nucleic acid therapeutics*, 24(4):283–290, 2014.

[48] Virginia Arechavala-gomeza, Maria Kinali, Lucy Feng, Michela Guglieri, Geraldine Edge, Marion Main, David Hunt, Jan Lehovsky, Volker Straub, Kate Bushby, Caroline A Sewry, Jennifer E Morgan, and Francesco Muntoni. Revertant fibres and dystrophin traces in Duchenne muscular dystrophy: Implication for clinical trials. *Neuromuscular Disorders*, 20(5):295–301, 2010.

[49] Q. L. Lu, G. E. Morris, S. D. Wilton, T. Ly, O. V. Artem'yeva, P. Strong, and T. a. Partridge. Massive idiosyncratic exon skipping corrects the nonsense mutation in dystrophic mouse muscle and produces functional revertant fibers by clonal expansion. *Journal of Cell Biology*, 148(5):985–995, 2000.

[50] C J Klein, D D Coovert, D E Bulman, P N Ray, J R Mendell, and a H Burghes. Somatic reversion/suppression in Duchenne muscular dystrophy (DMD): evidence supporting a frame-restoring mechanism in rare dystrophin-positive fibers. *American journal of human genetics*, 50(5):950–959, 1992.

[51] P A MacLennan and R H Edwards. Protein turnover is elevated in muscle of mdx mice in vivo. *The Biochemical journal*, 268:795–797, 1990.

[52] Aurélie Goyenvalle, Graziella Griffith, Arran Babbs, Samir El Andaloussi, Kariem Ezzat, Aurélie Avril, Branislav Dugovic, Rémi Chaussenot, Arnaud Ferry, Thomas Voit, Helge Amthor, Claudia Bühr, Stefan Schürch, Matthew J a Wood, Kay E Davies, Cyrille Vaillend, Christian Leumann, and Luis Garcia. Functional correction in mouse models of muscular dystrophy using exon-skipping tricyclo-DNA oligomers. *Nature Medicine*, 21(3), 2015.

[53] Rosie Z Yu, Brenda Baker, Alfred Chappell, Richard S Geary, Ellen Cheung, and Arthur a Levin. Development of an ultrasensitive noncompetitive hybridization-ligation enzyme-linked immunosorbent assay for the determination of phosphorothioate oligodeoxynucleotide in plasma. *Analytical biochemistry*, 304(1):19–25, 2002.

[54] K Ezzat, Y Aoki, T Koo, G McClorey, L Benner, A Coenen-Stass, L O'Donovan, T Lehto, A Garcia-Guerra, J Nordin, A F Saleh, M Behlke, J Morris, A Goyenvalle, B Dugovic, C Leumann, S Gordon, M J Gait, S El-Andaloussi, and M JA Wood. Self-Assembly into Nanoparticles Is Essential for Receptor Mediated Uptake of Therapeutic Antisense Oligonucleotides. *Nano Letters*, 2015.

[55] T. P. Prakash, M. J. Graham, J. Yu, R. Carty, a. Low, a. Chappell, K. Schmidt, C. Zhao, M. Aghajan, H. F. Murray, S. Riney, S. L. Booten, S. F. Murray, H. Gaus, J. Crosby, W. F. Lima, S. Guo, B. P. Monia, E. E. Swayze, and P. P. Seth. Targeted delivery of antisense oligonucleotides to hepatocytes using triantennary N-acetyl galactosamine improves potency 10-fold in mice. *Nucleic Acids Research*, 42(13):8796–8807, 2014.

Supplemental Information

Preliminary in vivo tests were performed in *mdx52* mice, which were subjected to one single eye intravenous injection of 10mg/kg of LNA-modified oligonucleotide and sacrificed ten to eleven weeks after the injection. Functional and behavioral testing of the mice was performed and compared with WT C57BL/6J mice. Preliminary tests and data from articles showed that no significant differences were obtained in the functional and behavioral tests, especially regarding the RotaRod test, subsequently we decided to perform, in the following experiments, only some of the functional and behavioral tests (Grip Strength, OpenField and Wire Hang) and perform the analysis of the molecular and biochemical parameters (Figure 14). In this experiment, we also detected dystrophin-positive fibers in treated *mdx52* tibialis anterior muscle (Figure 13), which could indicate skipping of exon 51, however skipping of exon 51 was no observable at the RNA level neither protein via Western Blot.

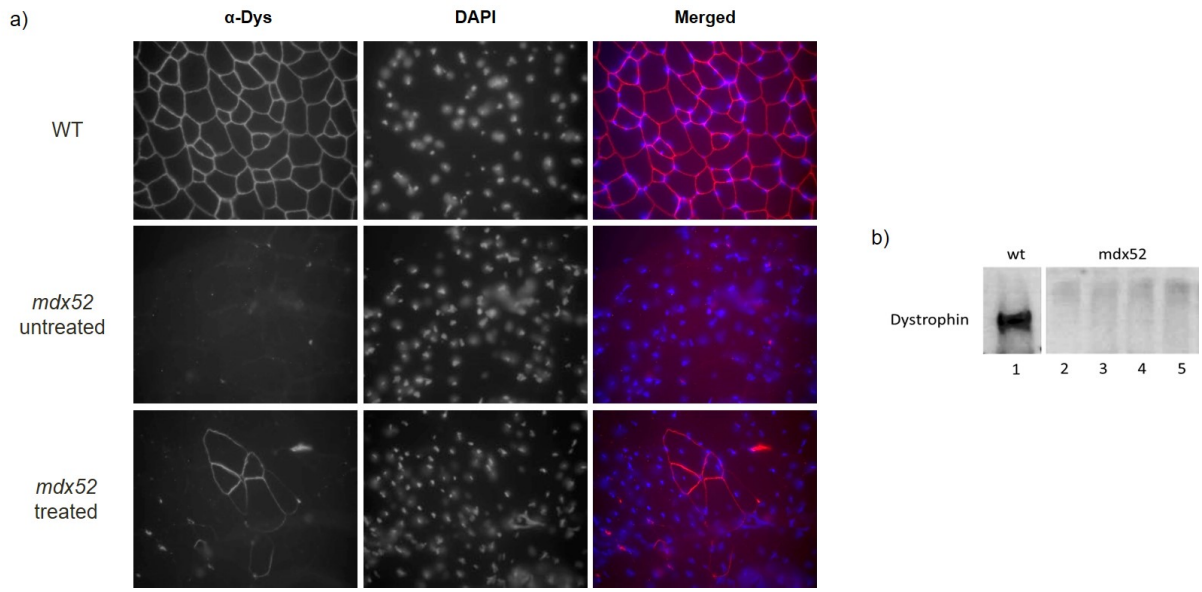

Figure 13: **Efficacy of exon 51 skipping in treated *mdx52* mice injected once with eye intravenous injection of 10mg/kg**. a) Immunohistochemical staining of dystrophin in transverse cryosections of tibialis anterior muscle of treated and untreated *mdx52* mice and C57BL/6J mice, used as a positive control, these observations were made using a Leica DM5000B Widefield Fluorescence Microscope. b) Western blotting analysis to detect the expression of dystrophin in gastrocnemius (GC) muscle (2 – untreated and 3-5 – treated *mdx52* mice). Protein extracts from GC of C57BL/6J were used as a positive control. Representative data are shown in the Immunohistochemistry and Western Blot results.

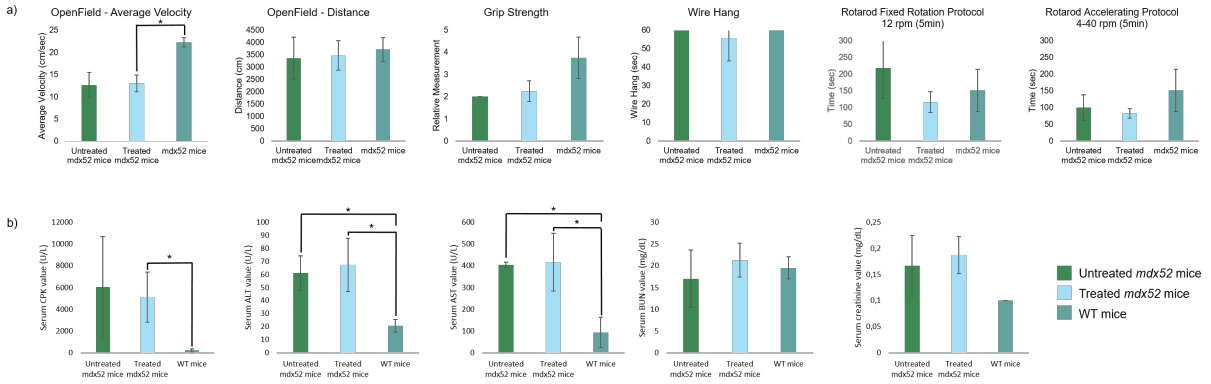

Figure 14: **Exon 51 skipped dystrophin did not ameliorate skeletal muscle function in *mdx52* mice injected once with eye intravenous injection of 10mg/kg**. a) Functional and behavioral testing of the mice was performed using Grip Strength (relative measurement of 1 to 4, being 1 - weak and 4 – strong, given by the handler of the force exerted by a mouse on a grille when its tail is pulled backwards), Wire Hang (sec), OpenField (cm/sec and sec) and RotaRod (sec), in treated and untreated *mdx52* mice with one intravenous injection of 10mg/kg of LNA-modified oligonucleotide. C57BL/6J were used as a control. Data (untreated n=3, treated n=8 and wt=4) are presented as mean ±SD. b) Measurement of biochemical markers [creatine phosphokinase – CPK (U/L), blood urea nitrogen – BUN (mg/dL), creatinine (mg/dL), aspartate transaminase - AST(U/L) and alanine transaminase – ALT (U/L)] levels.

The short (16mer) LNA-AON targeted to induce skipping of exon 51 in dystrophin mRNA used for *in vitro* and *in vivo* splicing modulation is represented in Figure 15.

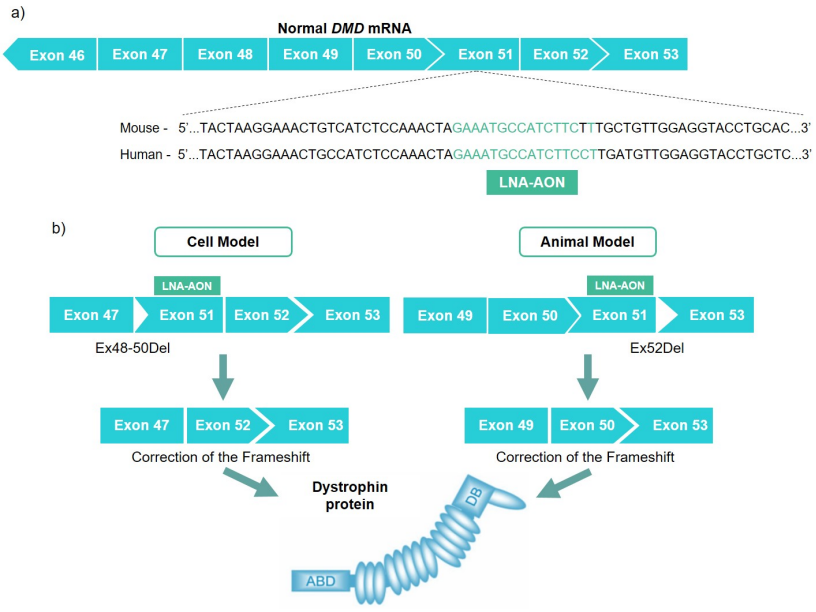

Figure 15: Schematic representations of a) the hybridization site of the LNA-AON tested that targets skipping of exon 51 in the DMD transcript and b) the myoblast derived cell lines from patients and the *mdx52* mouse deletions in the mRNA of the DMD gene (deletion of exons 48-50 and exon 52, respectively), that lead to out-of-frame products. Exon 51 skipping with the LNA-AON restores the reading frame of the dystrophin mRNA, correcting the frameshift and subsequently leading to the production of a truncated but partially functional dystrophin protein.