Conclusão do Caso de Estudo 1: "A herança da Maria"

A Biologia Molecular como base de desenvolvimento de novas terapias

Margarida Gama-Carvalho, FML 2010

Desenvolvimento de novas terapias

- Prova de conceito – ensaio em modelos celulares
- Sistema de administração
- Ensaio em modelos animais (toxicidade e eficácia)
- Ensaios clínicos (várias fases, nº crescente de doentes)
  - Toxicidade/segurança
  - Eficácia da resposta
  - Eliminação

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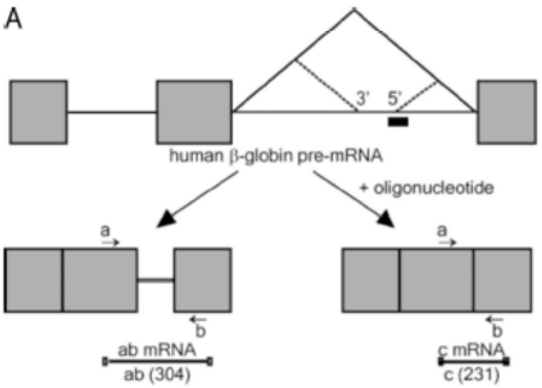
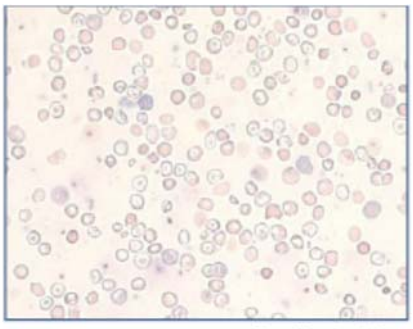
Modelação de splicing na β-talassémia

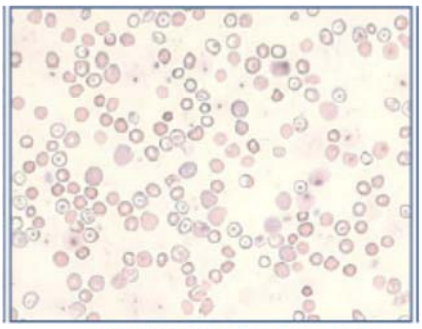
PubMed: thalassemia [and] splicing [and] therapy = 26 results

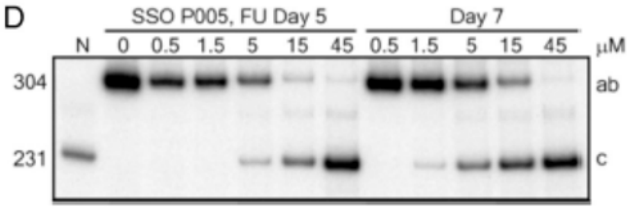
- Dominski Z, Kole R (1993) Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. Proc Natl Acad Sci U S A 90(18): 8673-8677.
- Sierakowska H, Sambade MJ, Agrawal S, Kole R (1996) Repair of thalassemic human beta-globin mRNA in mammalian cells by antisense oligonucleotides. Proceedings of the National Academy of Sciences of the United States of America 93(23): 12840-12844.
- Gorman L, Suter D, Emerick V, Schumperli D, Kole R (1998) Stable alteration of pre-mRNA splicing patterns by modified U7 small nuclear RNAs. Proc Natl Acad Sci U S A 95(9): 4929-4934.
- Suter D, Tomasini R, Reber U, Gorman L, Kole R, Schumperli D (1999) Double-target antisense U7 snRNAs promote efficient skipping of an aberrant exon in three human beta-thalassemic mutations. Hum Mol Genet 8(13): 2415-2423.
- Suwanmanee T, Sierakowska H, Fucharoen S, Kole R (2002a) Repair of a splicing defect in erythroid cells from patients with beta-thalassemia/HbE disorder. Mol Ther 6(6): 718-726.
- Suwanmanee T, Sierakowska H, Lacerra G, Svasti S, Kirby S, Walsh CE, Fucharoen S, Kole R (2002b) Restoration of human beta-globin gene expression in murine and human IVS2-654 thalassemic erythroid cells by free uptake of antisense oligonucleotides. Mol Pharmacol 62(3): 545-553.
- Svasti S, Suwanmanee T, Fucharoen S, Moulton HM, Nelson MH, Maeda N, Smithies O, Kole R (2009) RNA repair restores hemoglobin expression in IVS2-654 thalassemic mice. Proc Natl Acad Sci U S A 106(4): 1205-1210.

Svasti S, et al (2009) RNA repair restores hemoglobin expression in IVS2-654 thalassemic mice.









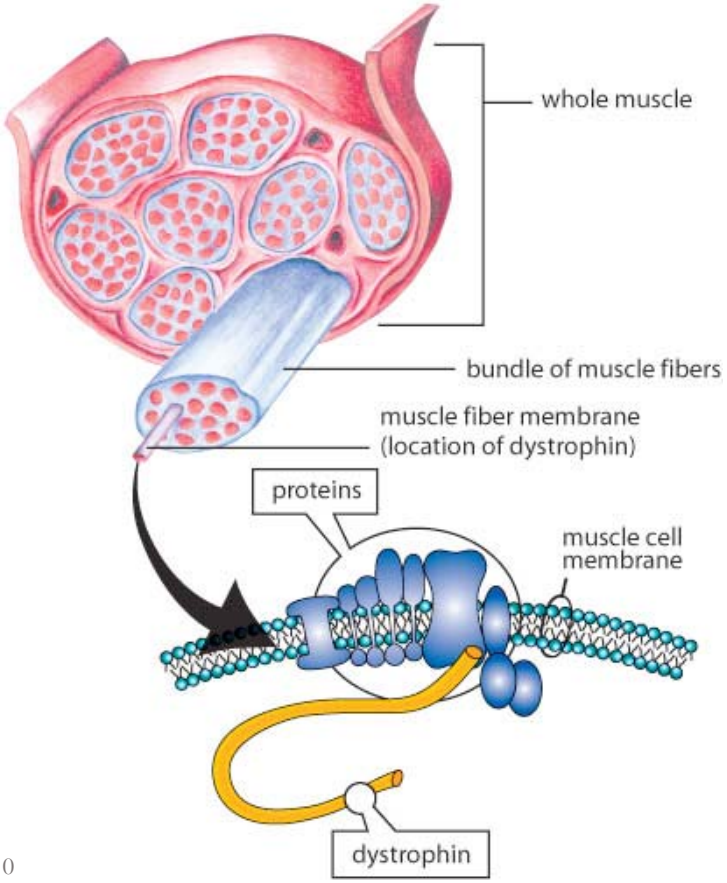
Porque não há ainda ensaios clínicos??

Distrofia Muscular de Duchenne

- Doença ligada ao cromossoma X
- Perda de função da proteína distrofina
- Atrofia muscular grave, com sintomas a surgirem por volta dos 5 anos e morte até aos 20 anos
  - Variante mais suave: Distrofia Muscular de Becker

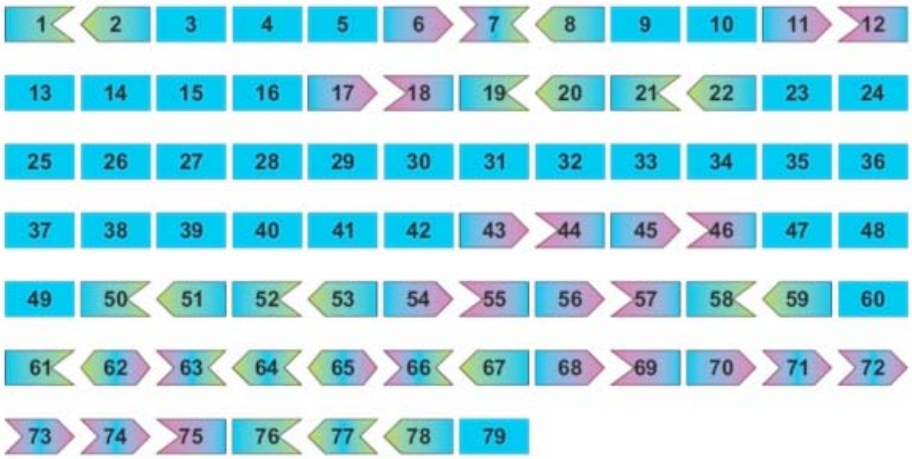
Distrofina:

- Função



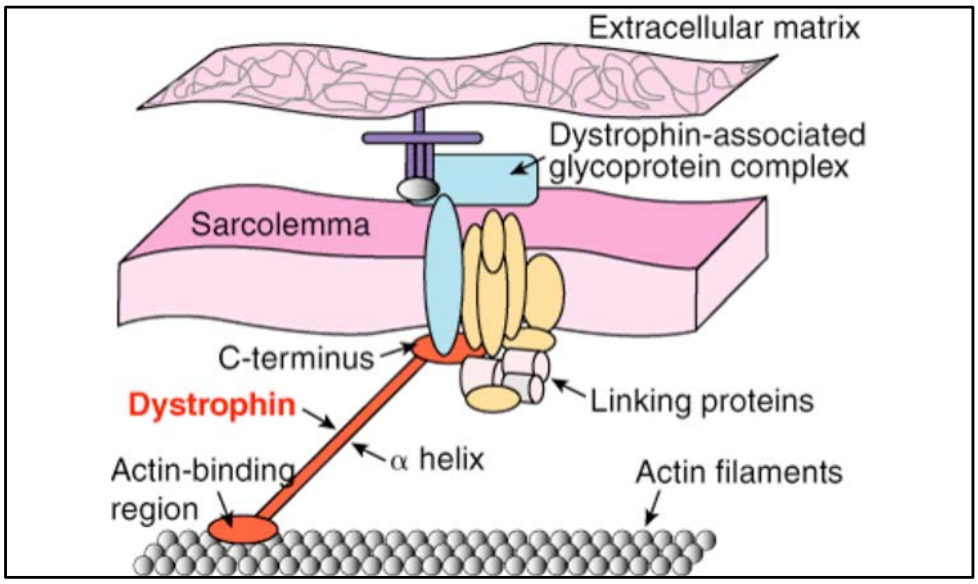
Distrofina

- Gene e proteína

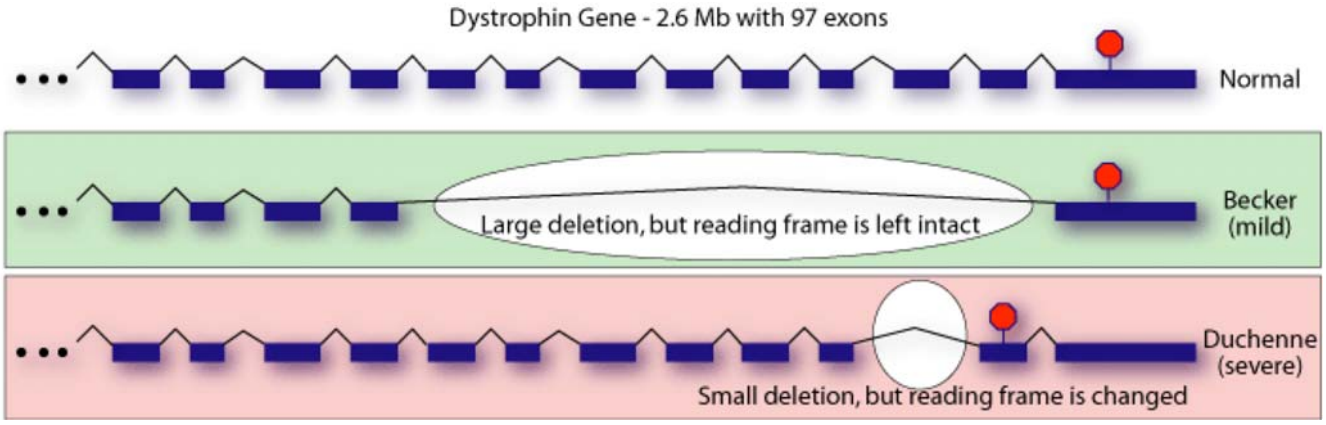


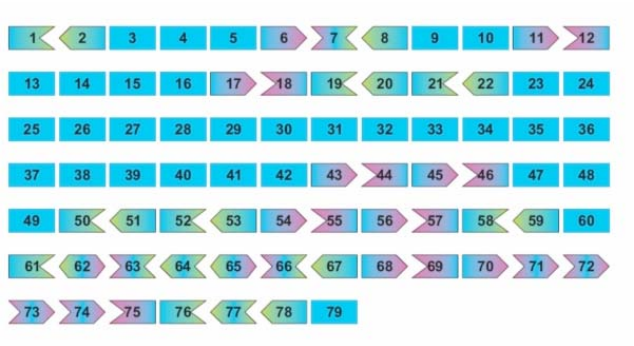
Distrofina

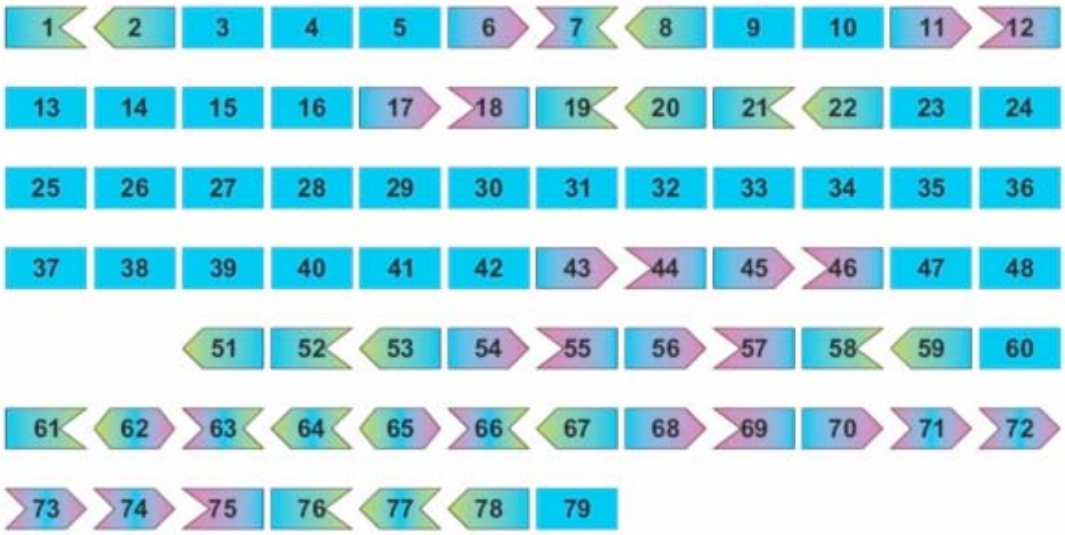
- Estrutura modular da proteína



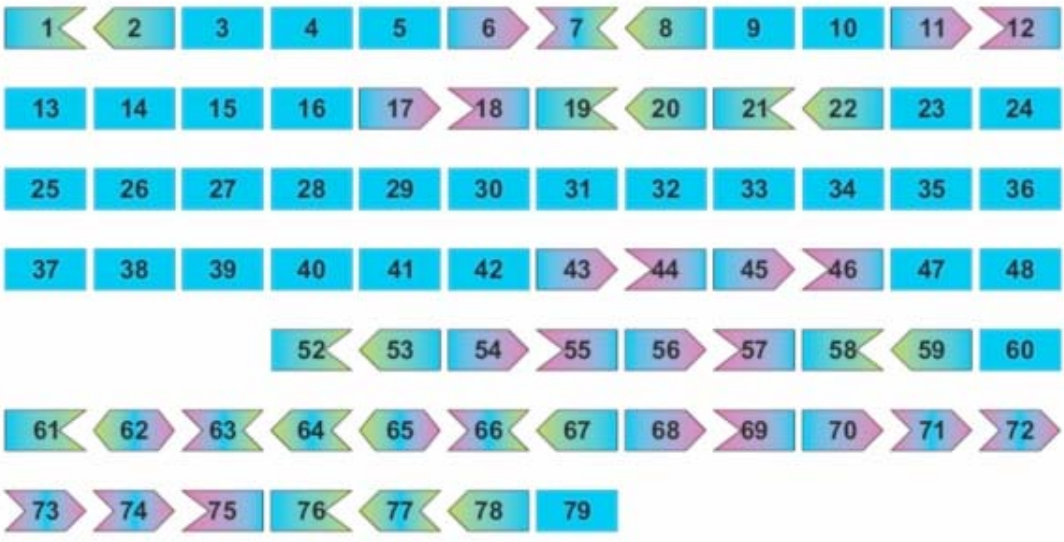
Mutações causadoras de DMD e BMD



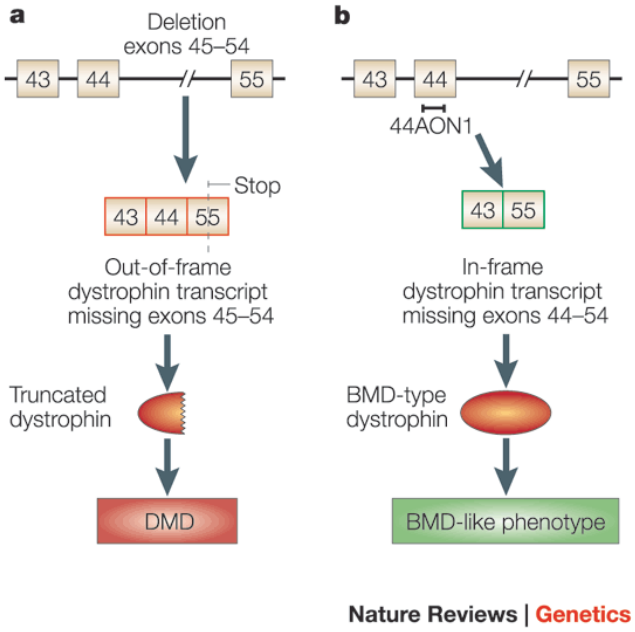




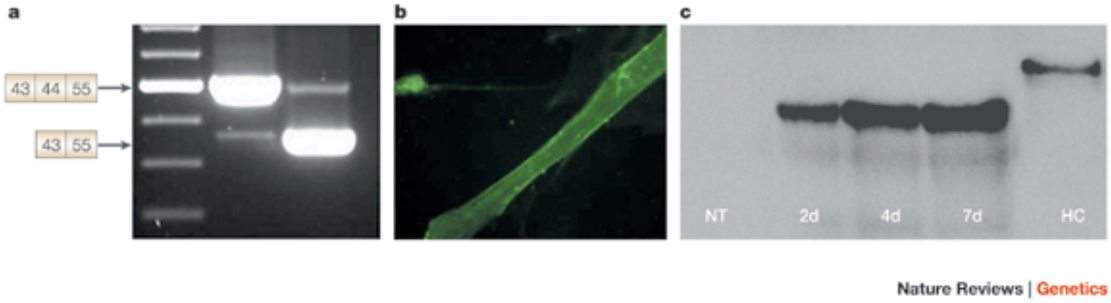
Exon skipping as a treatment for DMD



Regulação do splicing na DMD



Estudos celulares de exon-skipping



Lancet Neurol 2009; 8: 918–28

Local restoration of dystrophin expression with the morpholino oligomer AVI-4658 in Duchenne muscular dystrophy: a single-blind, placebo-controlled, dose-escalation, proof-of-concept study

Maria Kinali*, Virginia Arechavala-Gomeza*, Lucy Feng, Sebahattin Cirak, David Hunt, Carl Adkin, Michela Guglieri, Emma Ashton, Stephen Abbs, Petros Nihoyannopoulos, Maria Elena Garralda, Mary Rutherford, Caroline Mcculley, Linda Popplewell, Ian R Graham, George Dickson, Matthew JA Wood, Dominic J Wells, Steve D Wilton, Ryszard Kole, Volker Straub, Kate Bushby, Caroline Sewry, Jennifer E Morgan, Francesco Muntoni

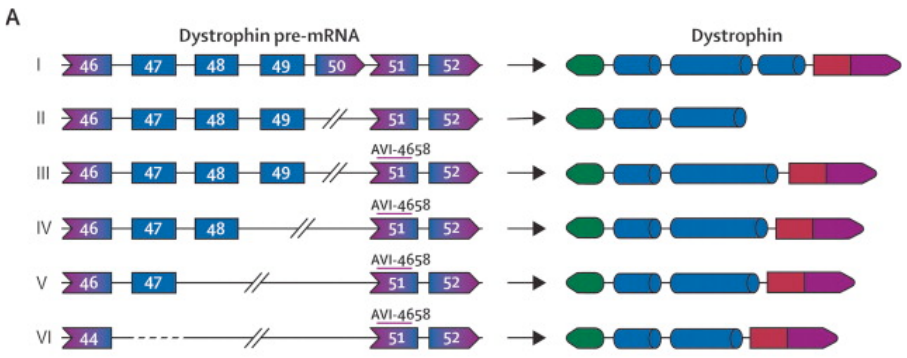
Summary

Background Mutations that disrupt the open reading frame and prevent full translation of *DMD*, the gene that encodes dystrophin, underlie the fatal X-linked disease Duchenne muscular dystrophy. Oligonucleotides targeted to splicing elements (splice switching oligonucleotides) in *DMD* pre-mRNA can lead to exon skipping, restoration of the open reading frame, and the production of functional dystrophin in vitro and in vivo, which could benefit patients with this disorder.

Methods We did a single-blind, placebo-controlled, dose-escalation study in patients with DMD recruited nationally, to assess the safety and biochemical efficacy of an intramuscular morpholino splice-switching oligonucleotide (AVI-4658) that skips exon 51 in dystrophin mRNA. Seven patients with Duchenne muscular dystrophy with deletions in the open reading frame of *DMD* that are responsive to exon 51 skipping were selected on the basis of the preservation of their extensor digitorum brevis (EDB) muscle seen on MRI and the response of cultured fibroblasts from a skin biopsy to AVI-4658. AVI-4658 was injected into the EDB muscle; the contralateral muscle received saline. Muscles were biopsied between 3 and 4 weeks after injection. The primary endpoint was the safety of AVI-4658 and the secondary endpoint was its biochemical efficacy. This trial is registered, number NCT00159250.

Estudo prévio dos doentes

- Confirmação da mutação por sequenciação completa do gene
- Estado do músculo
- Confirmação da ausência de expressão de variantes de splicing
- Confirmação da resposta ao morfolino in vitro



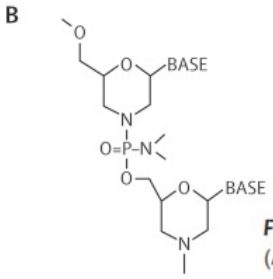
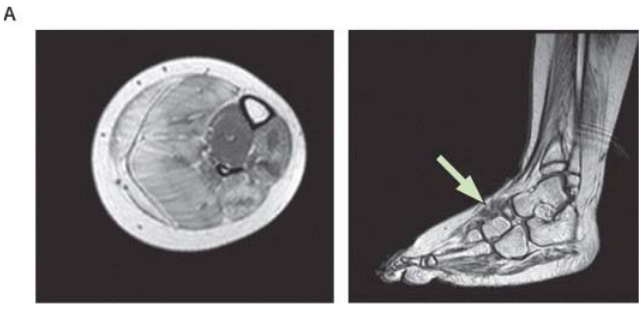


***Figure 1*: Deletions and predicted results of exon skipping in the patients who were studied**
(A) Pre-mRNA transcripts and dystrophin protein products from full length *DMD*, in patients with Duchenne muscular dystrophy, and predicted protein sequences after exon skipping. (I) The normal dystrophin gene produces the full length dystrophin product. (II) Patients 1 and 2 had a deletion in exon 50 that disrupts the open reading frame, leading to a truncated and unstable dystrophin. (III) Skipping of exon 51 restores the reading frame, producing a truncated but functional dystrophin that lacks exons 50 and 51. (IV) Patient 7 is missing exons 49 and 50. (V) Patients 3 and 4 are missing exons 48–50. (VI) Patients 5 and 6 are missing exons 45–50. All the truncated dystrophins produced after skipping of exon 51 are missing the hinge 3 region and some of the rod domain but have been associated with the milder BMD phenotype.[9,10] (B) Structure of the phosphorodiamidate morpholino modification of the antisense oligomer.

The Lancet Neurology 2009; 8:918-928



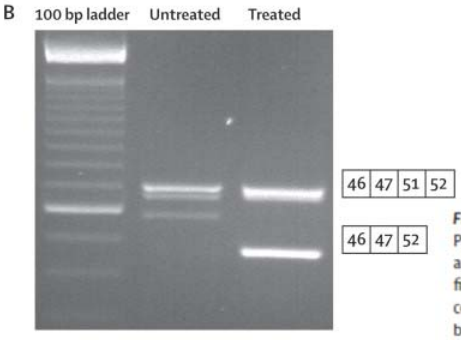
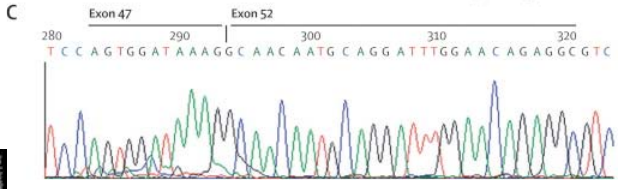


***Figure 2*: Procedure for prescreening of patients before injection of AVI-4658.**
Patient 3 is shown as an example; similar results were obtained for all patients. (A) Transverse MRI of the lower leg and coronal MRI of the extensor digitorum brevis muscle (arrow) confirmed the suitability of the muscle. (B) Skin fibroblasts from all patients were forced into myogenic differentiation and treated with an AVI-4658 congener to confirm exon skipping and dystrophin production. RT-PCR analysis shows two bands: the high molecular weight band corresponds to the unskipped transcript (including exons 46, 47, 51, and 52) and the low molecular weight band corresponds to the transcript fragment with size specific skipping of exon 51. (C) Exon 51 skipping was confirmed by sequencing.



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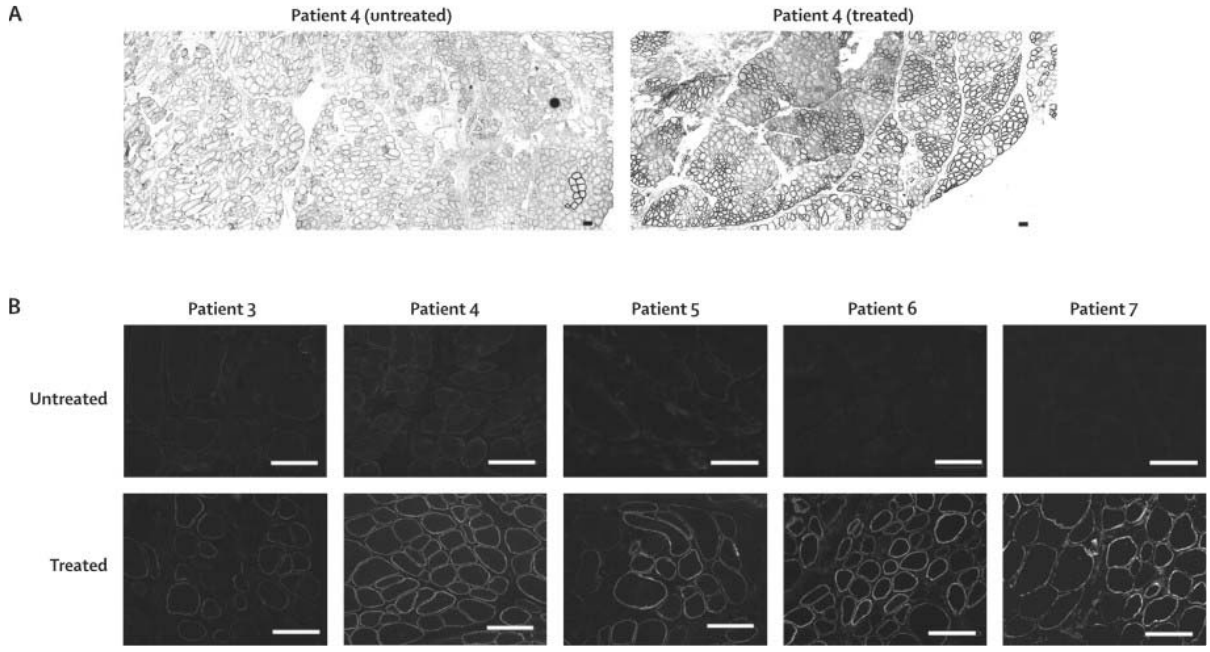

***Figure 3*: Dystrophin expression in patients treated with high-dose AVI-4658**
Transverse sections of treated and contralateral EDB muscles that were immunostained for dystrophin with MANDYS106. (A) Low-power micrograph of a whole section taken with ×10 objective lens shows widespread expression of dystrophin in fibres from the treated muscle in patient 4. (B) Higher magnification (×20 objective lens) of dystrophin immunolabelling in treated and untreated sections in patients 3–7. Scale bars=100 μm.

	Untreated		Treated	
	Total	Positive	Total	Positive
3	443	21 (5%)	377	182 (49%)
4	662	2 (<1%)	792	623 (79%)
5	475	2 (<1%)	263	116 (44%)
6	554	5 (1%)	404	264 (65%)
7	405	3 (<1%)	262	164 (63%)

***Table 3*: Dystrophin expression in muscle myofibres in patients 3–7, who were treated with high-dose AVI-4658**

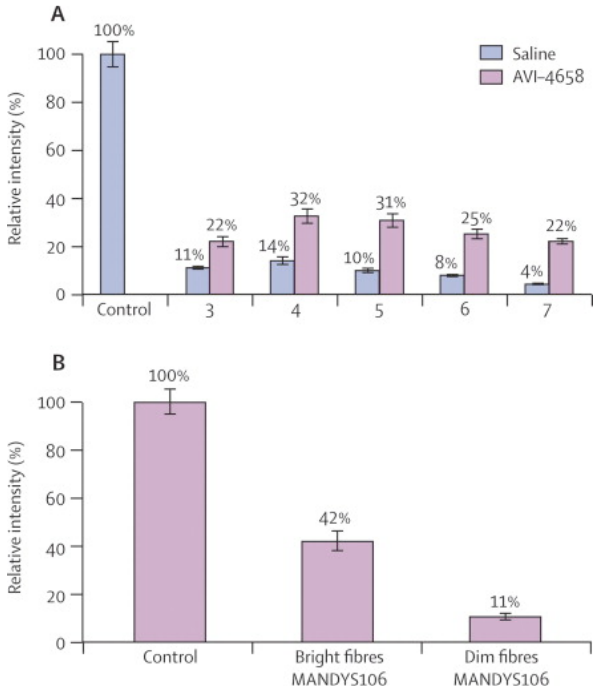

***Figure 4*: Intensity of dystrophin expression in patients treated with high-dose AVI-4658 relative to control**
(A) Mean random intensity measurements. (B) Measurement of mean dystrophin intensity in positive fibres: intensity measurements exclusively targeted to 100 dystrophin-positive and 100 dystrophin-negative fibres within the same area in patient 4. Bars are SEM.

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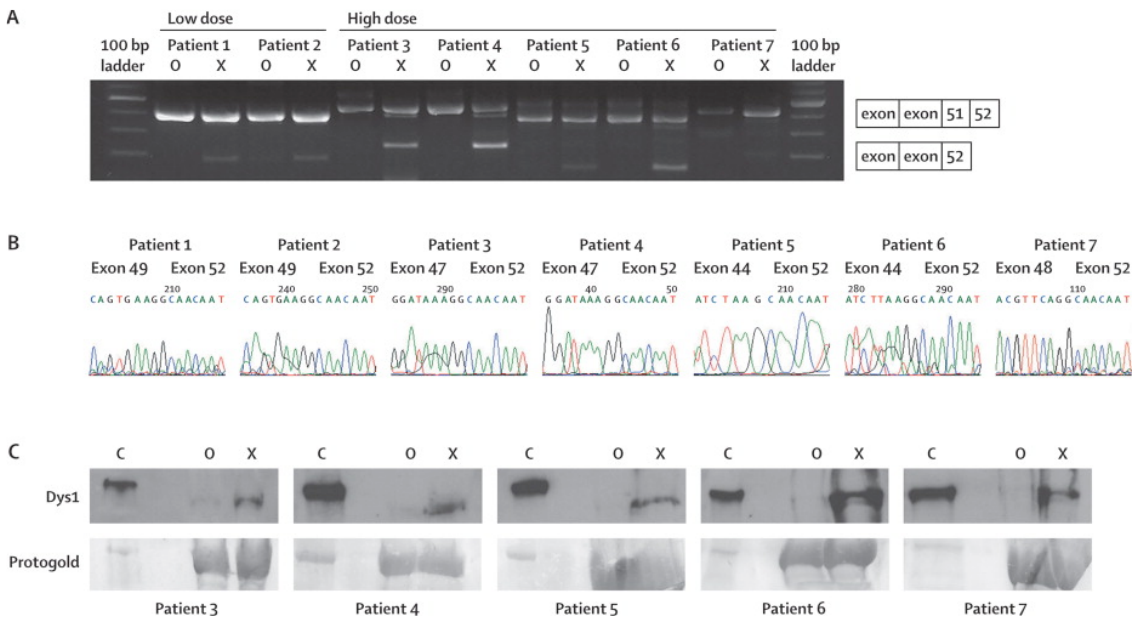

***Figure 5*: Exon 51 skipping in amplified RNA from treated muscles**

(A) RT-PCR analysis of RNA extracted from treated (X), untreated (O), and control (C) muscle sections detects shorter transcript fragments in the treated muscles, with sizes that correspond to the specific skipping of exon 51. (B) Exon 51 skipping was confirmed by sequencing. (C) Western blot analysis of homogenates of treated and untreated muscle (20x10 µm sections) and control muscle (2x10 µm sections [to avoid overexposure]) shows dystrophin expression in extracts from the control muscles (C) and treated (X) extensor digitorum brevis but not in the contralateral muscles (O). Loading was monitored with protogold. Low dose=0·09 mg AV-4658. High dose=0·9 mg AV-4658.

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