✔ Duchenne Muscular Dystrophy

✔ miRNAs and Duchenne Muscular Dystrophy

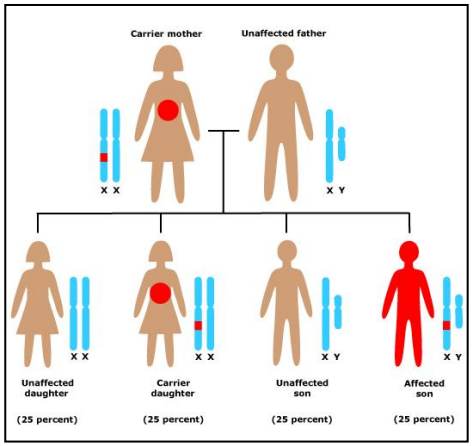
✔ Use of miRNAs as biomarkers or diagnostic

✔ microRNA therapeutics: state of the art (miRNA decoy system to enhance exon skipping efficacy)

La Distrofia Muscolare di Duchenne (DMD)

Venne scoperta ed analizzata, nel 1868, dal neurologo francese **Guillaume-Benjamin-Amand Duchenne**

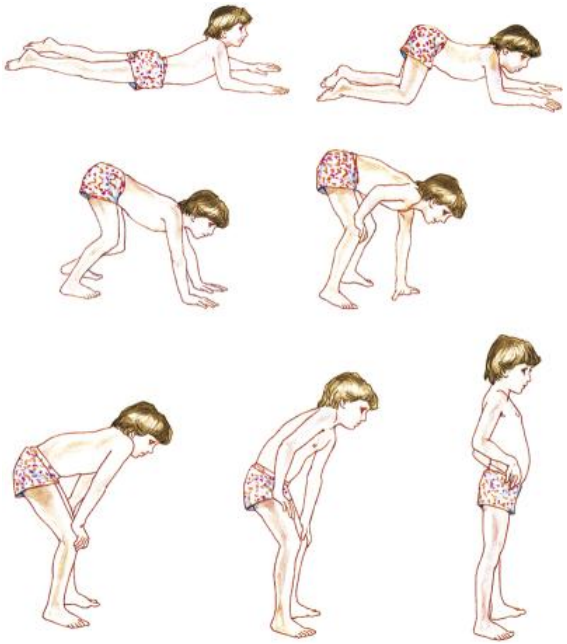
✔ E' una malattia recessiva legata al cromosoma X



✔ Colpisce 1 su 3500 bambini nati vivi

✔ E' caratterizzata dall'assenza di una proteina, la Distrofina, essenziale per la stabilizzazione della membrana cellulare della fibra muscolare

Decorso della malattia



La DMD è caratterizzata da progressiva degenerazione muscolare. A causa di questa marcata riduzione del tono muscolare diventa difficile camminare. Per alzarsi da terra il bambino compie movimenti caratteristici: spingendosi sulle mani, le appoggia sul pavimento in seguito le pone sulle ginocchia e sulle cosce e raggiunge la posizione eretta che ha però un atteggiamento lordico. Il decorso della malattia è rapido e porta all'uso della sedia a rotelle entro il 13 anno di vita.

Campanelli d'allarme

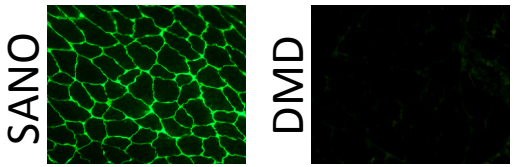
Diagnosi prenatale in caso di precedenti familiari

Riscontro casuale, nei bambini al di sotto dei 3 anni, di elevati livelli di creatin chinasi (CK) nel sangue

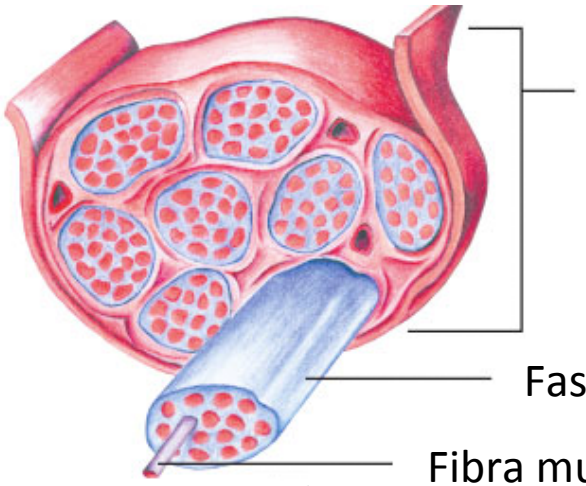
Ridotta abilità fisica rispetto ai coetanei

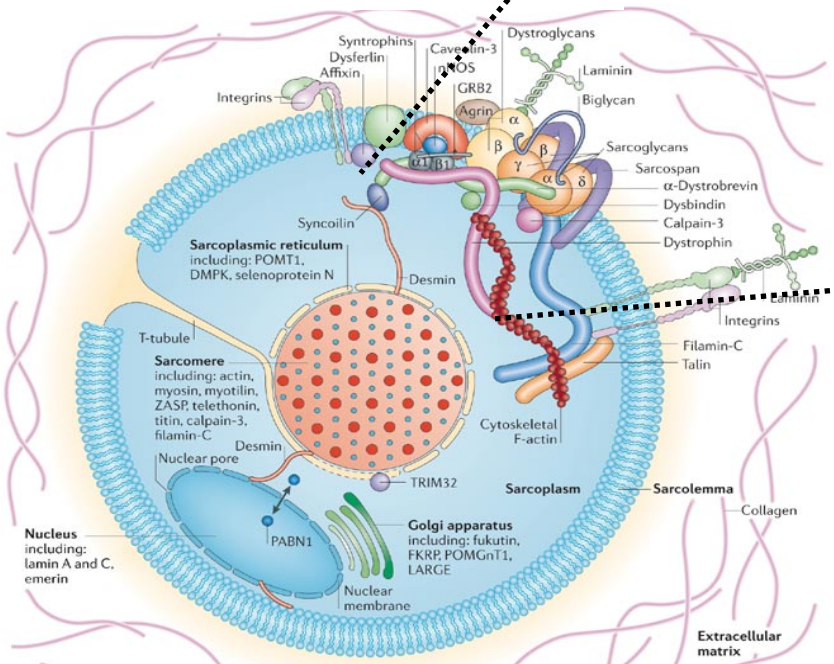
Diagnosi

1- Assenza di distrofina in biopsie muscolari



2- Diagnosi molecolare da prelievo di sangue





Davies and Nowak *Nature Reviews Molecular Cell Biology* **7**, 762–773 (2006)

La Distrofina

È codificata dal gene più grande del nostro genoma, la sua trascrizione dura 16 ore (1 mm)

DNA = 2,5 x 10^6 bp

mRNA = 14 Kb

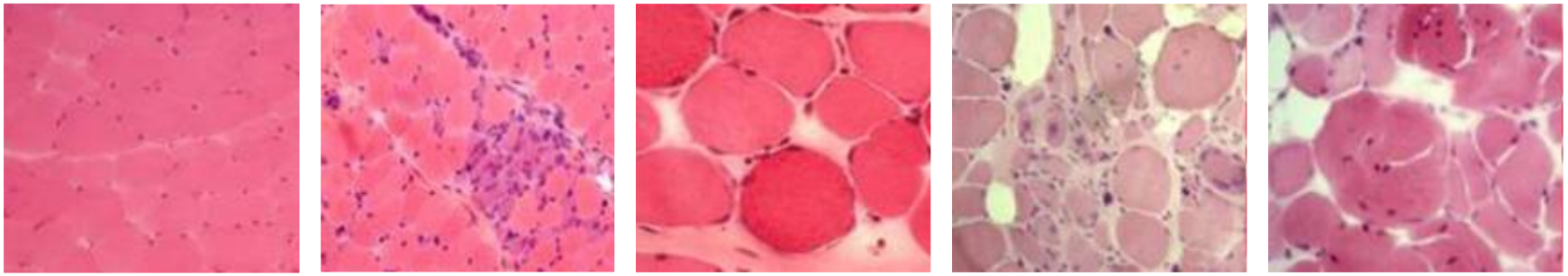
Proteina= 427 KDa

30% dei pazienti hanno una anamnesi familiare negativa (insorgenza di nuove mutazioni).

Duchenne Muscular Dystrophy (DMD)

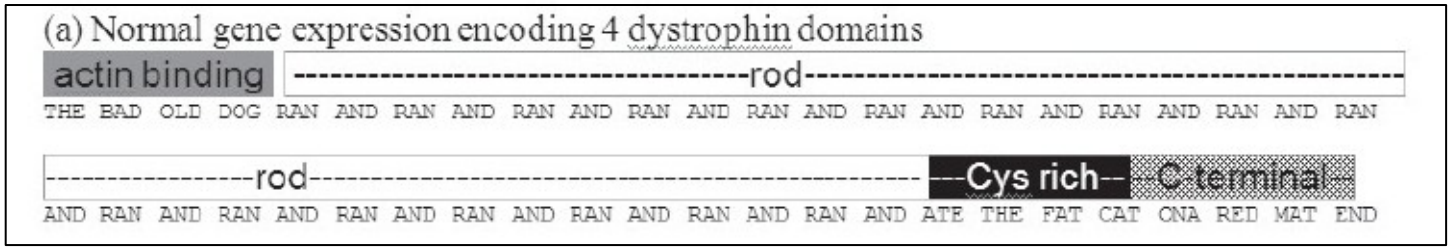
is a severe disorder characterized by rapid progression of muscle degeneration leading to loss of ambulation and death.

Histopathology of a Duchenne muscle

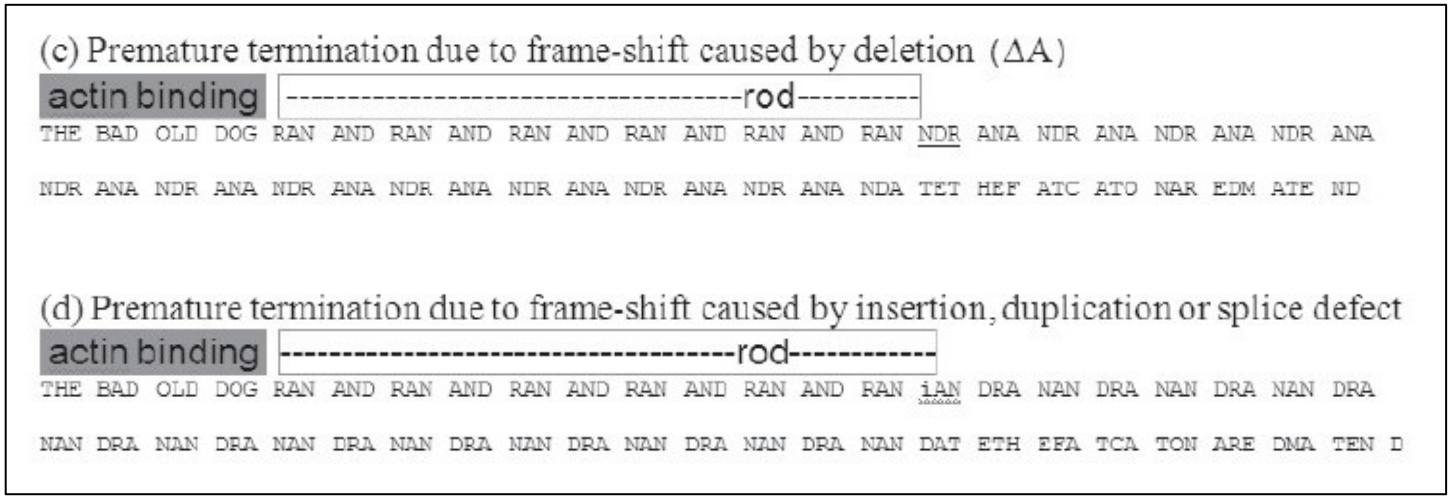


Dystrophin

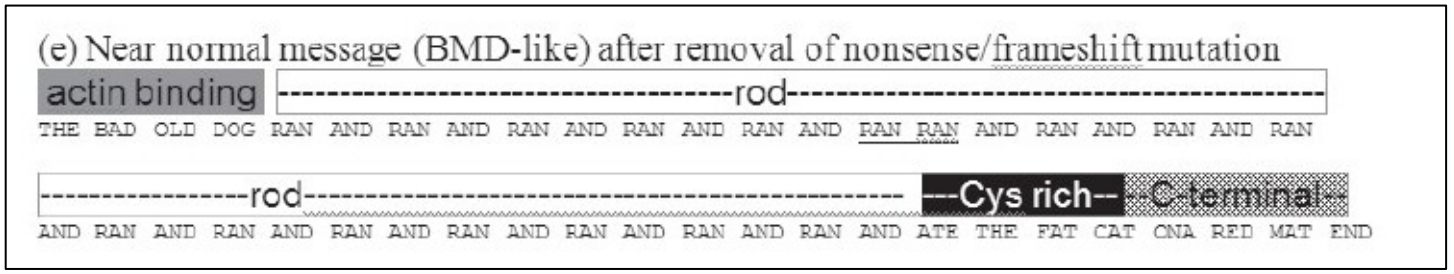
Healty



DMD



BMD



Approcci alla terapia delle distrofie muscolari

Farmaci - recupero del registro di lettura (PTC), stimolare l'exon skipping o la produzione di proteine d'interesse come l'utrofina.
Limiti: somministrazione continua

Terapia genica - sviluppo di nuovi vettori capaci di trasferire il gene mancante ai nuclei delle fibre muscolari.
Limiti: gene troppo grande - difficoltà a raggiungere efficacemente tutti i distretti muscolari

Terapia cellulare - ricostituire un tessuto funzionale fornendo cellule satellite o cellule staminali (mesoangioblasti).
Limiti: limitata capacità proliferativa e migratoria delle cellule satelliti. Necessità di cellule autologhe.

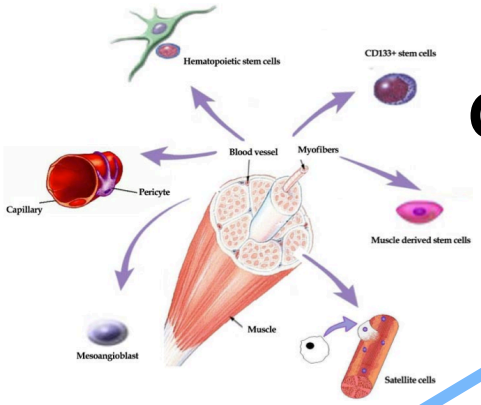
THERAPY FOR DUCHENNE MUSCULAR DYSTROPHY

Drug therapy

Read through

PTC-124
(Ataluren)

Cell therapy



Gene therapy

Introduction of a functional, recombinant version of the dystrophin gene

AAV-mediated gene delivery

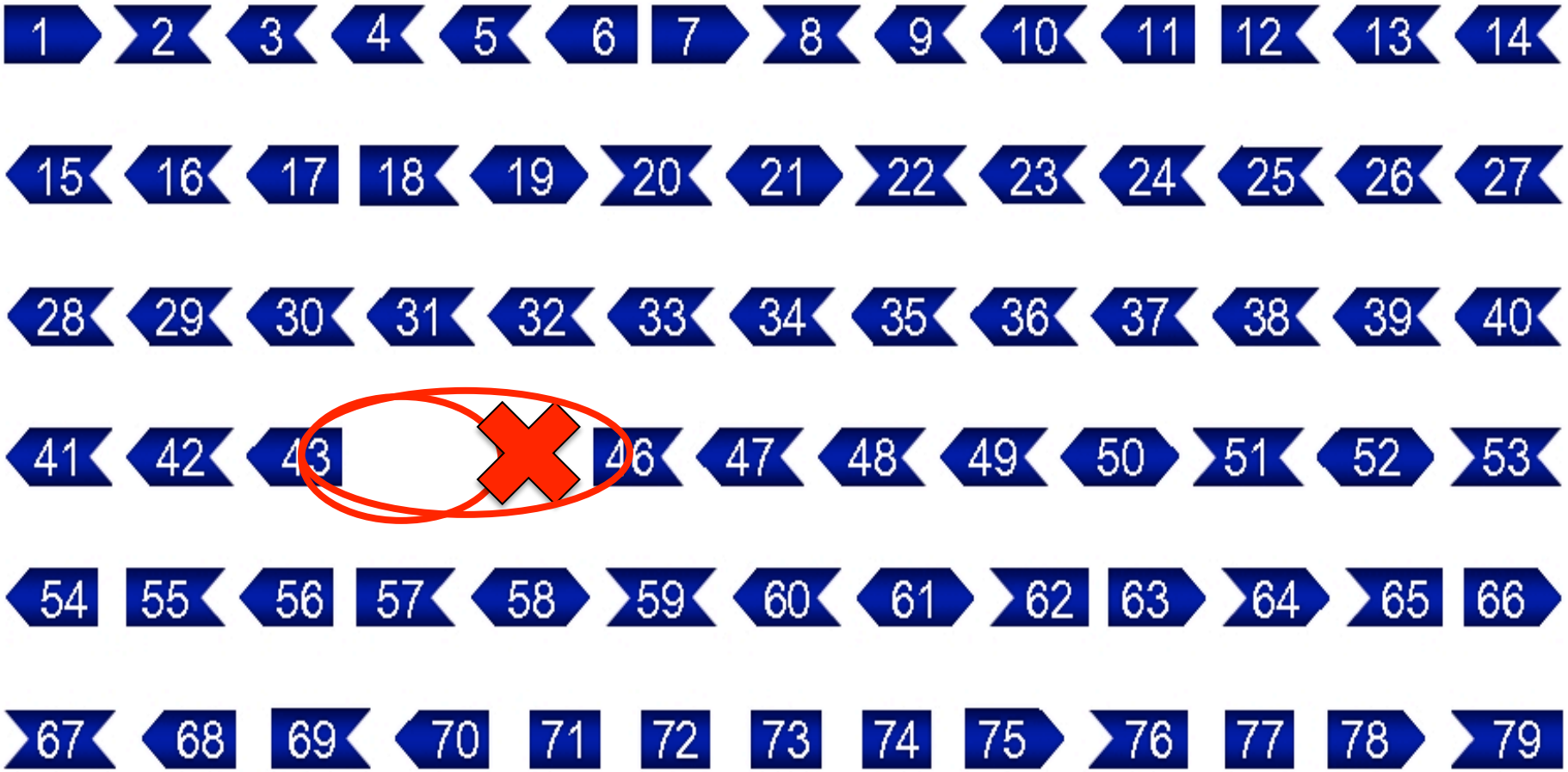
Modification of dystrophin pre-mRNA splicing

Exon skipping

TRIAL CLINICI

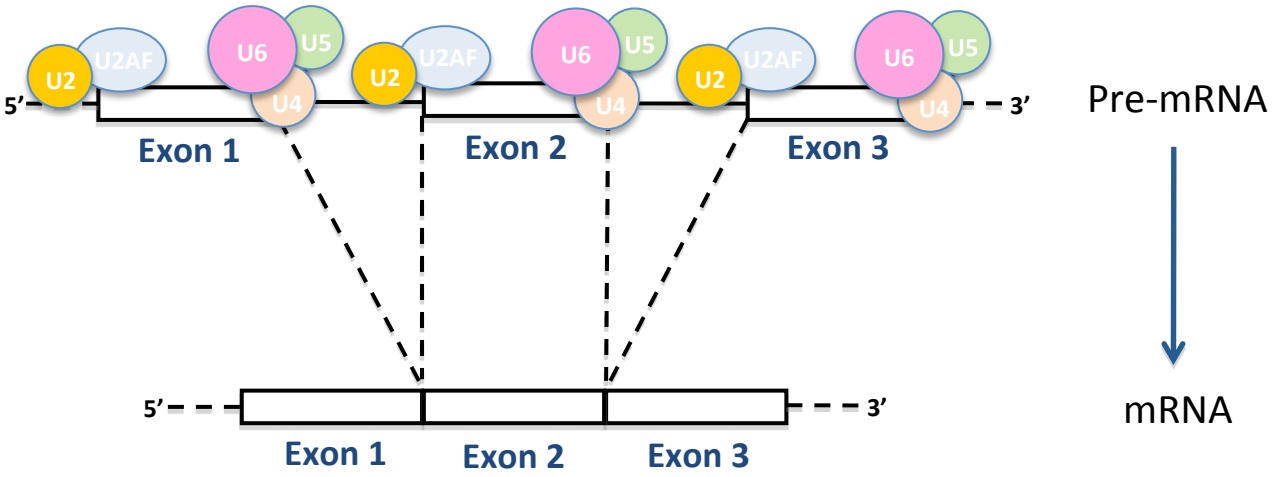
Category	Interventions	Phase (ClinicalTrials.gov)
Drug	**Myostatin blocking**	
	MYO-029	Completed; not effective
	Read-through	
	PTC124	Completed; not effective
	Gentamicin	Completed; not effective
	Others	
	Pentoxifylline	Completed; not effective
	Idebenone	Phase III
	Ramipril vs. Carvedilol	Phase IV
	Coenzyme Q10 and prednisone	Phase III
	Coenzyme Q10 and lisinopril	Phase II/III
	Debio-025 (cyclosporine analogue)	Phase IIb
Cell therapy	**Satellite cells (myoblasts)**	Pending
	Mesoangioblasts	In preparation
	Induced pluripotent stem (iPS) cells	Experimental
Gene therapy	**Exon skipping** (systemic delivery)	
	PRO051 (2′-*O*-MePS AO)(exon 51 skipping)	Phase III
	PRO044 (2′-*O*-MePS AO)(exon 44 skipping)	Phase I/II
	AVI-4658 (PMO)(exon 51 skipping)	Phase IIb
	AAV vector	
	rAAV2.5-CMV-Mini-Dystrophin	Phase I*

The Exon 44 deletion

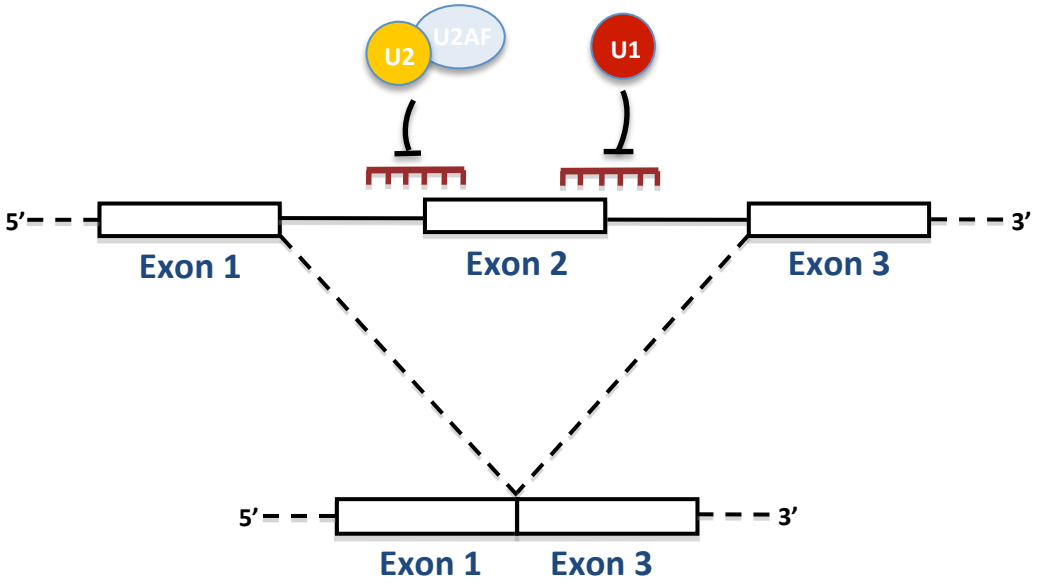


EXON SKIPPING

Exclusion of a specific exon from an mRNA sequence



EXON SKIPPING



Pre-mRNA

mRNA w/o exon 2

High levels
Low persistence
Immunogenicity

antisense oligonucleotides(AON)

expression constructs

Physiological levels
Long persistence
AAV delivery

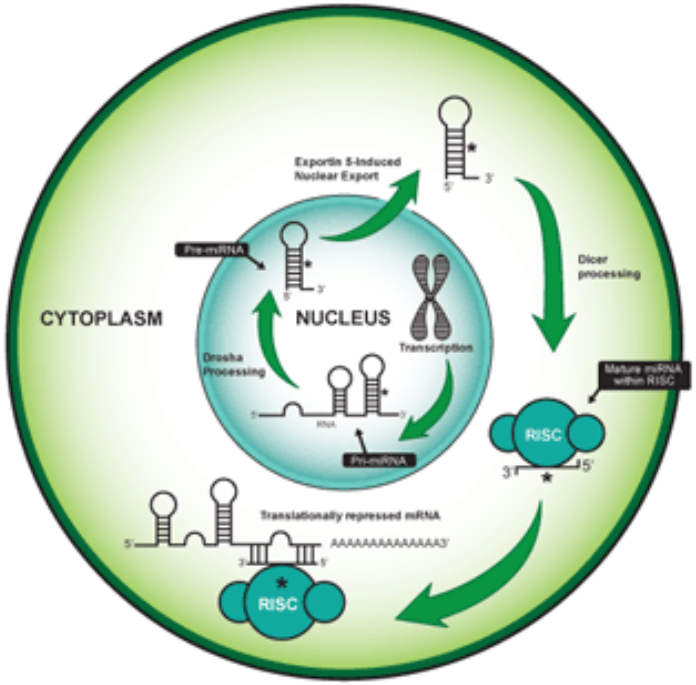
✔ Duchenne Muscular Dystrophy

✔ miRNAs and Duchenne Muscular Dystrophy

✔ Use of miRNAs as biomarkers or diagnostic

✔ microRNA therapeutics: state of the art (miRNA decoy system to enhance exon skipping efficacy)

miRNAs



miRNAs, that are small non coding RNA molecules measuring between 17 to 22 nucleotides, act as natural antisense molecules by negatively regulating the expression of genes with sequences that are complementary to the miRNAs.

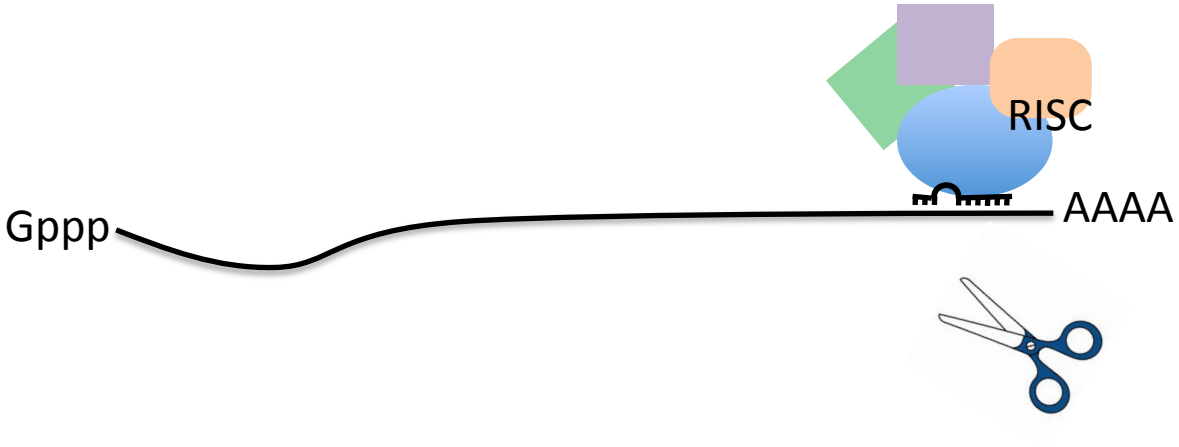
Each miRNA appears to regulate the expression of tens to hundreds of different genes. In many cases, it appears that miRNA regulate the expression of multiple, functionally related genes, making it possible to efficiently regulate the activities of specific cell processes.

Deregulation of miRNAs in pathological processes led to a constantly increasing amount of data connecting miRNAs to development of diseases such as cancer

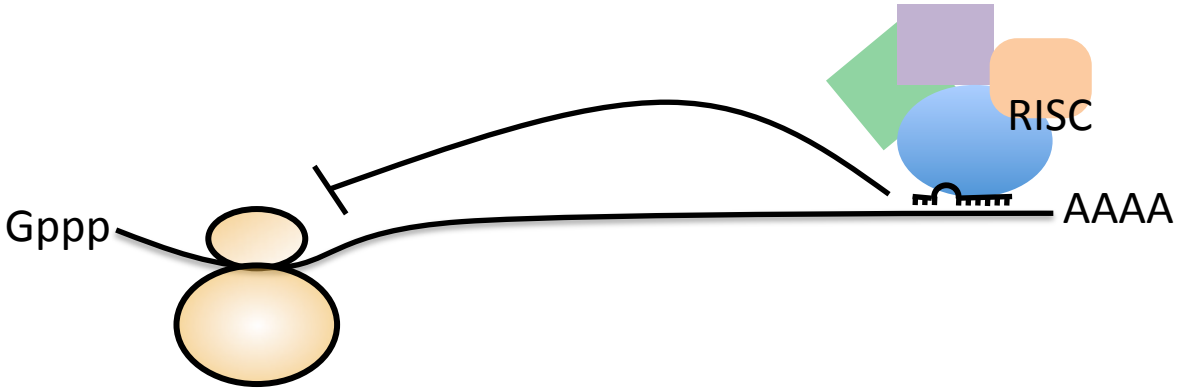
miRNA

Negative regulators of gene expression

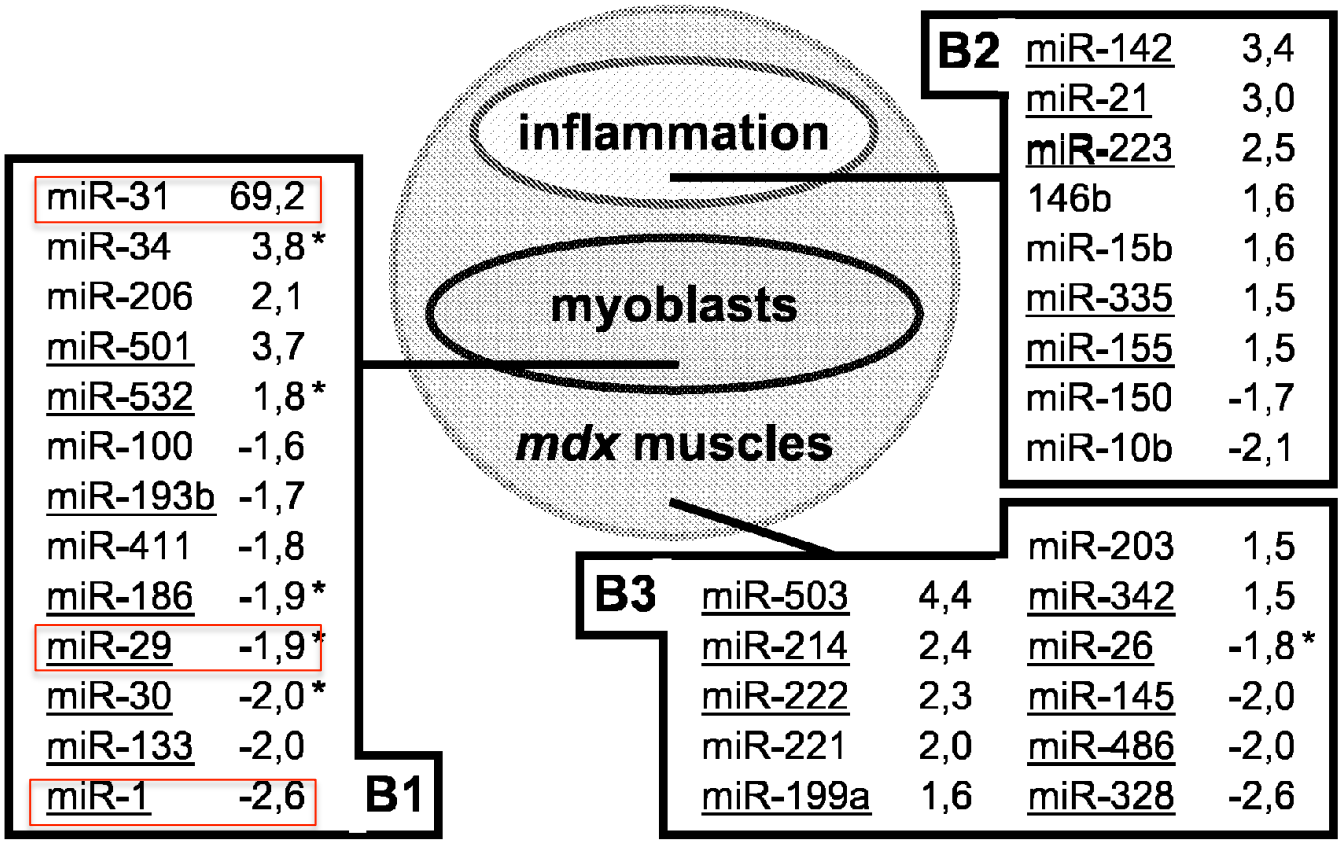
Target cleavage



Translational repression

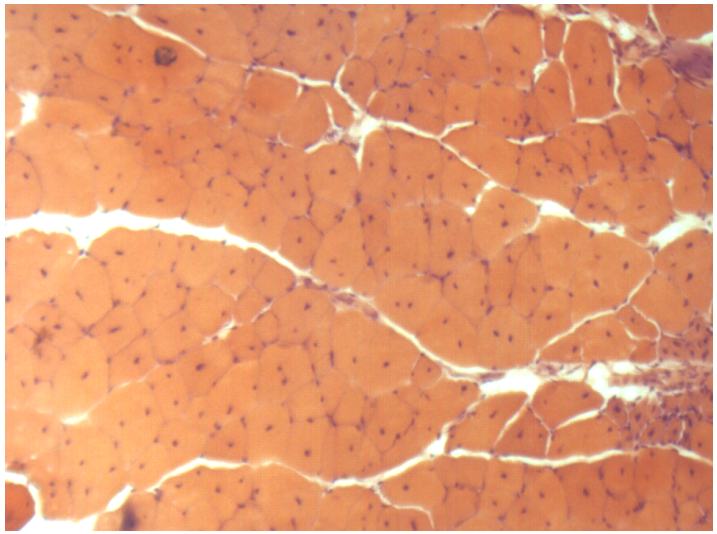


In Duchenne muscles several miRNAs are deregulated

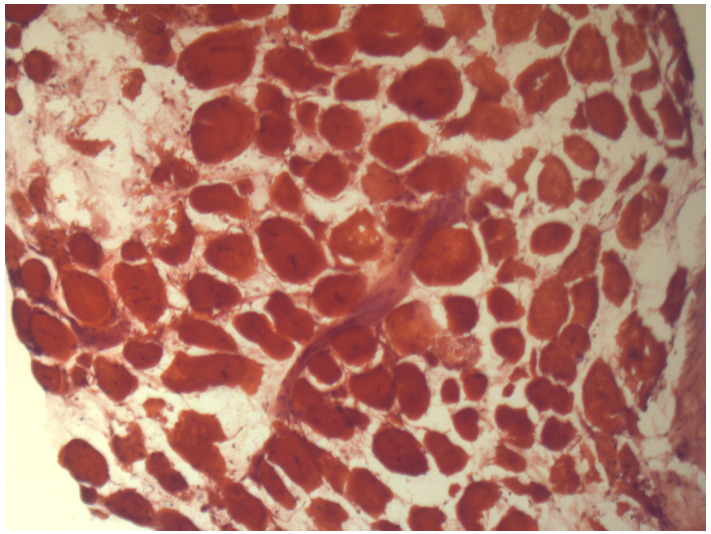


In Duchenne muscles several miRNAs are deregulated
- this explains several DMD pathogenetic traits -

WT



mdx



Inflammatory infiltration	↑	**miR-223**
Regeneration	↑	**miR-31, miR-206**
Oxidative stress	↓	**miR-1**
Fibro-adipogenic degeneration	↓	**miR-29**

mdx

Cacchiarelli et al., Cell Metabolism 2010

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miRNAs as biomarkers

By definition, a biomarker can be measured and evaluated as an indicator of normal biological processes, pathological processes, or pharmacological responses to therapeutic intervention.

The perfect candidate marker has to be:

- Specific to diseased organ or tissue. Able to differentiate pathologies
- Sensitive Rapid and significant release upon the development of pathology
- Robust Rapid, simple, accurate and inexpensive detection. Unconfounded by environment and unrelated conditions
- High predictive Long half-life in sample. Proportional to degree of severity of pathology
- Non-invasive Present in accessible fluid sample ease progression or therapeutic response), and easily
- Translatable from model systems to humans.

miRNAs as biomarkers

miRNAs were demonstrated to be robust against external impacts such as:

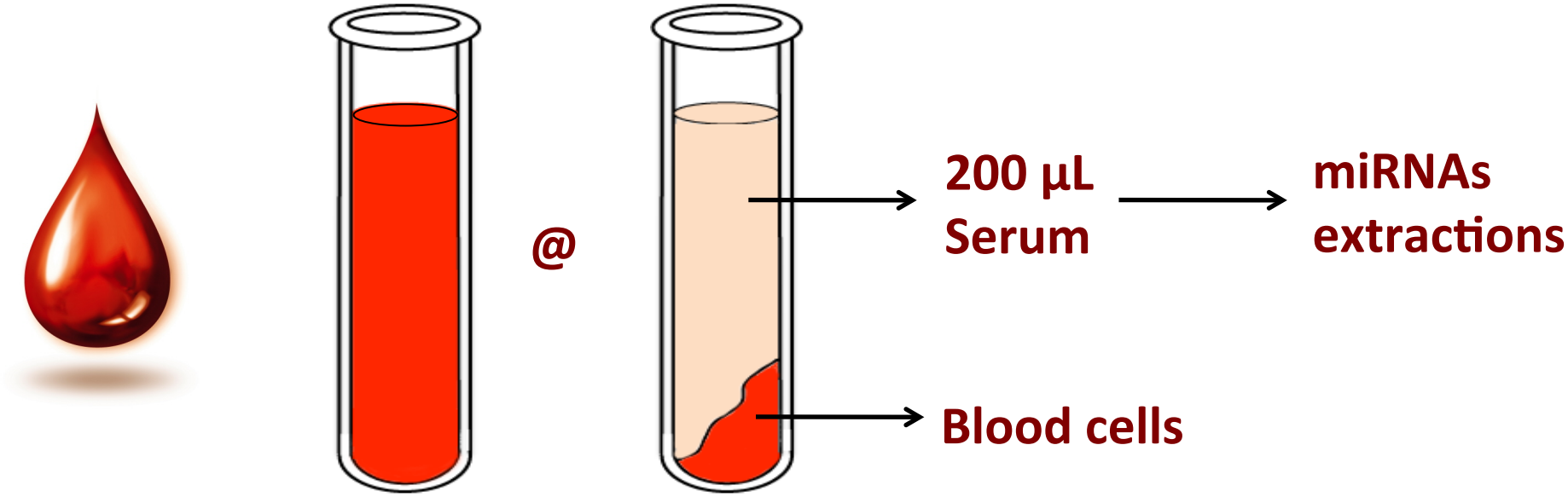
- enzymatic degradation
- freezing and thawing
- intense pH conditions
- not affected by different storage temperatures

and thus claimed to serve as an improved biomarker for several diseases.

miRNAs are detectable in almost all body fluids and excretions, including urine, feces, saliva, tear, ascetic, pleural, and amniotic fluid they could provide a new set of diagnostic tools for a variety of diseases.

miRNAs as serum biomarkers

Il siero sanguigno è il risultato di un liquido formato da plasma (fase liquida del sangue) senza fibrinogeno, fattore VIII, fattore V e protrombina.
Il fibrinogeno è una proteina solubile che nel processo di coagulazione del sangue viene convertita in fibrina, proteina non globulare ma filamentosa. Per ottenere un plasma senza fibrinogeno (quindi per ottenere il siero), in seguito al prelievo del sangue, si attende la coagulazione. In seguito, per centrifugazione del campione biologico, si separa la fase liquida del sangue dalla parte corpuscolare e si ottiene dunque il siero (wikipedia.



Muscle-specific miRNAs as serum biomarkers for:

-Duchenne Muscular Dystrophy (DMD)

is a monogenic disorder caused by mutations in the 2.5 Mb-long dystrophin gene (DMD). In the absence of dystrophin, muscle fibers become more sensitive to mechanical damage leading to muscle degeneration, chronic inflammatory response and increase in fibrosis, all of which exacerbate the dystrophic phenotype.

-Spinal muscular atrophy (SMA)

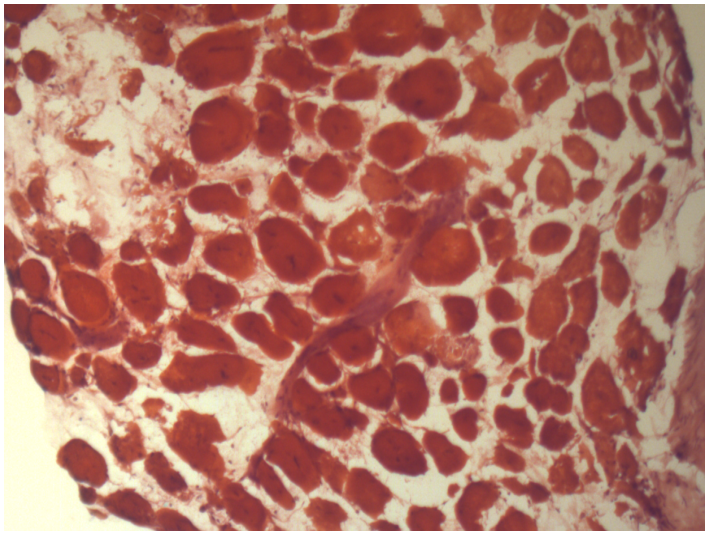
is an incurable autosomal recessive disease caused by a genetic defect in the SMN1 gene which codes SMN, a protein necessary for survival of motor neurons, and resulting in death of neuronal cells in the anterior horn of spinal cord and subsequent system-wide muscle wasting (atrophy).

-Amyotrophic Lateral Sclerosis (ALS)

is the most common form of the motor neuron diseases. The disorder is characterized by rapidly progressive weakness, muscle atrophy and fasciculations, muscle spasticity, difficulty speaking (dysarthria), difficulty swallowing (dysphagia), and decline in breathing ability.

Muscle miRNAs as biomarkers of muscle damage

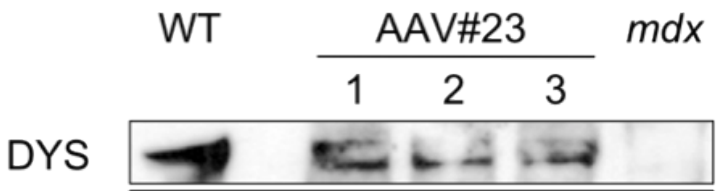
-Duchenne Muscular Dystrophy (DMD)

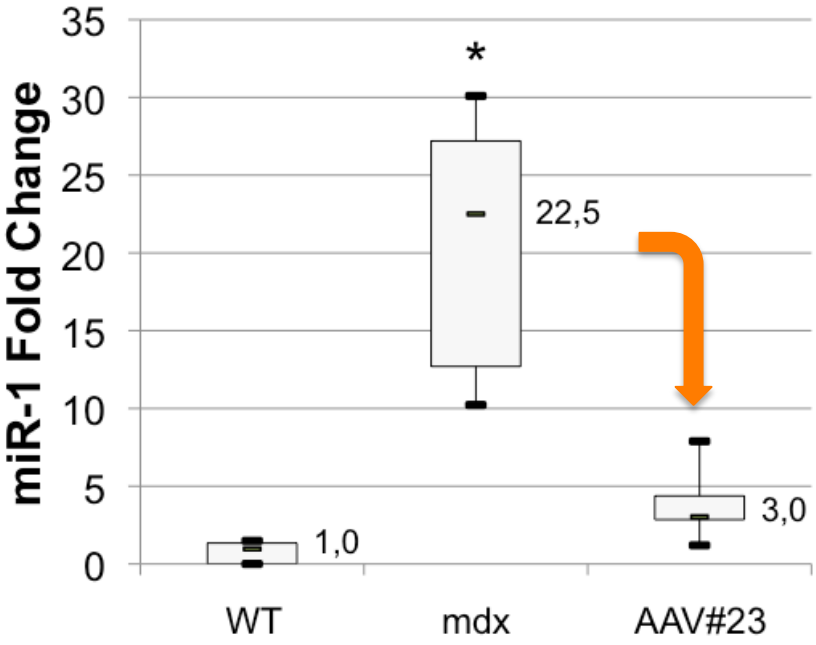


Muscle degeneration leads to leakage
of cellular components into the blood

Circulating muscle miRNAs to monitor gene therapy efficacy

(miR-1, miR-206, miR-133)



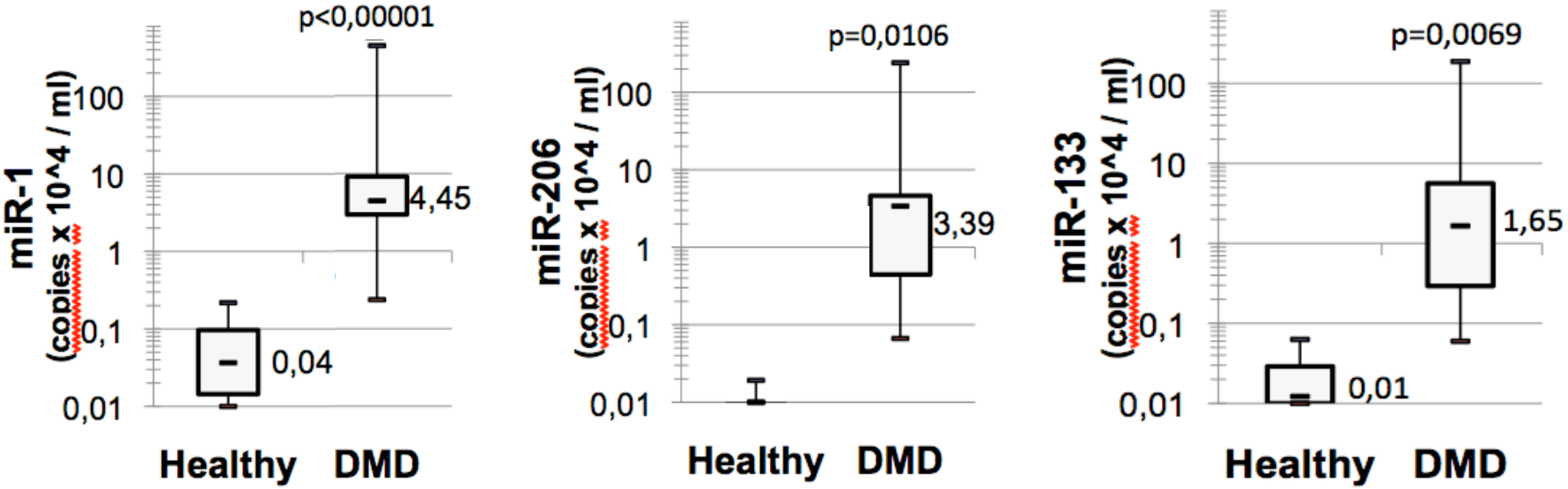


Therapeutic intervention leads to rescue of dystrophin…

…and decrease of miR-1 in the serum

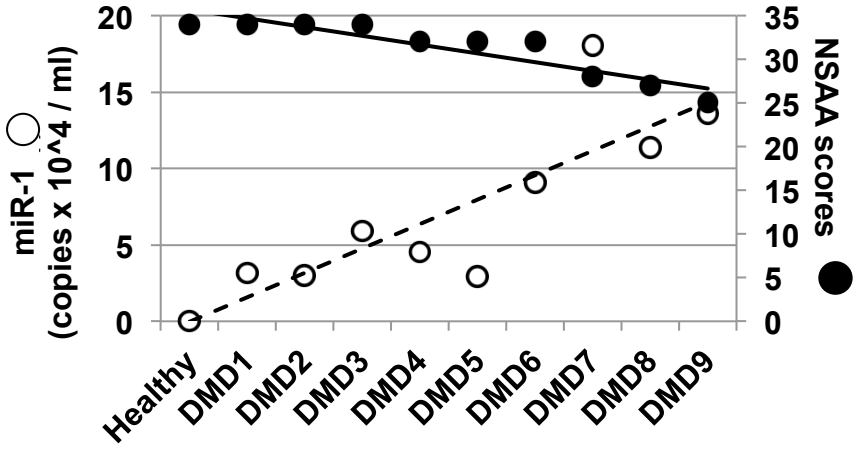
Muscle-specific miRNAs as serum biomarkers for DMD

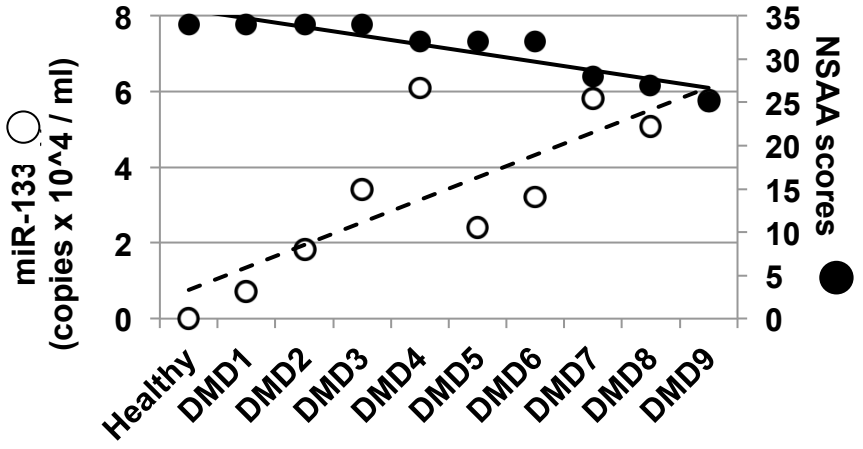
Differential amounts of muscle miRNAs in the sera of healthy versus dystrophic children

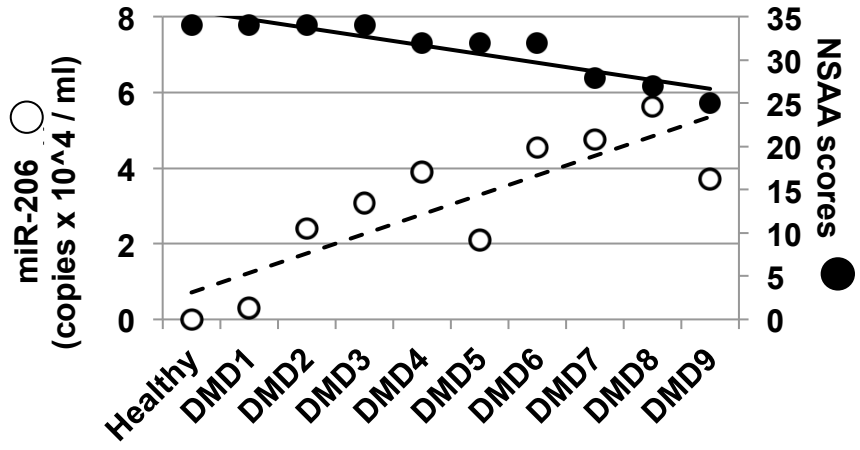


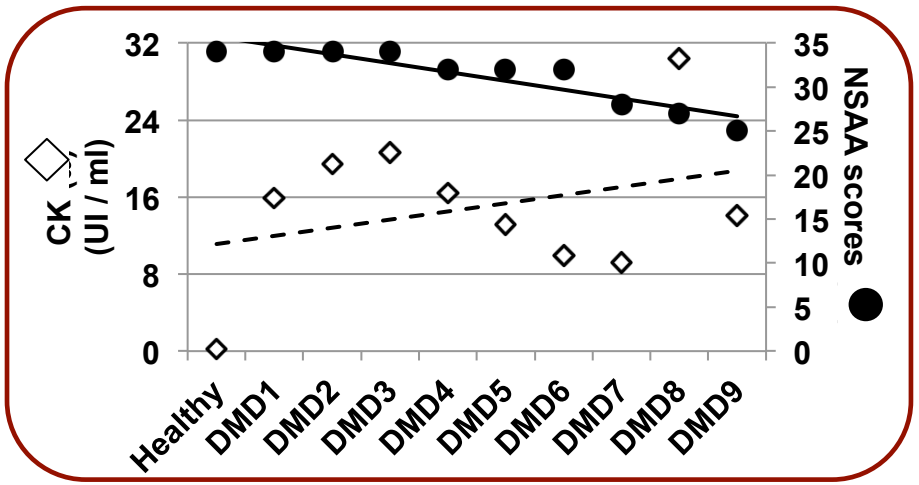
Cacchiarelli et al. 2010

Correlation between serum miRNAs and clinical assessments (North Star Ambulatory Assessment)





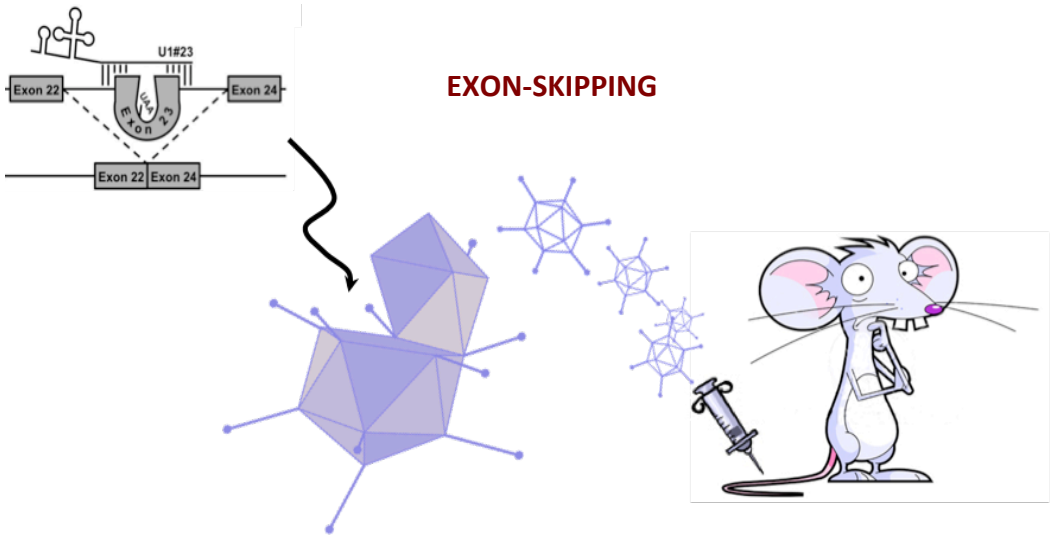


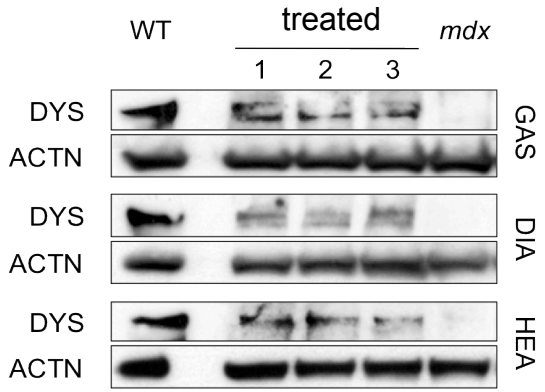


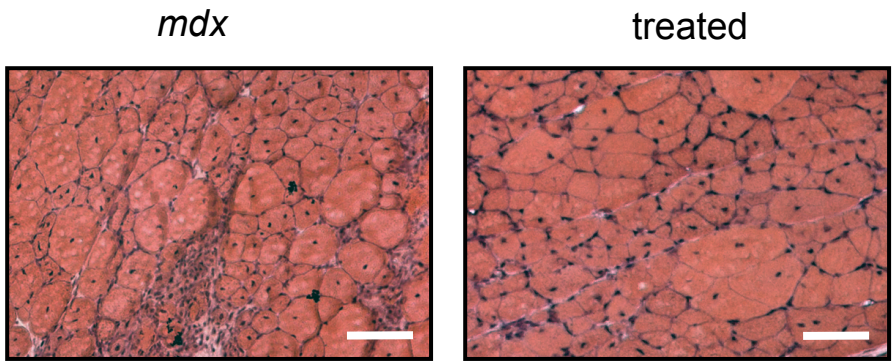
miRNAs levels correlate with the severity of the disease better than creatine kinase levels

Cacchiarelli et al. 2010

miRNAs as biomarkers for therapeutic outcome measurements

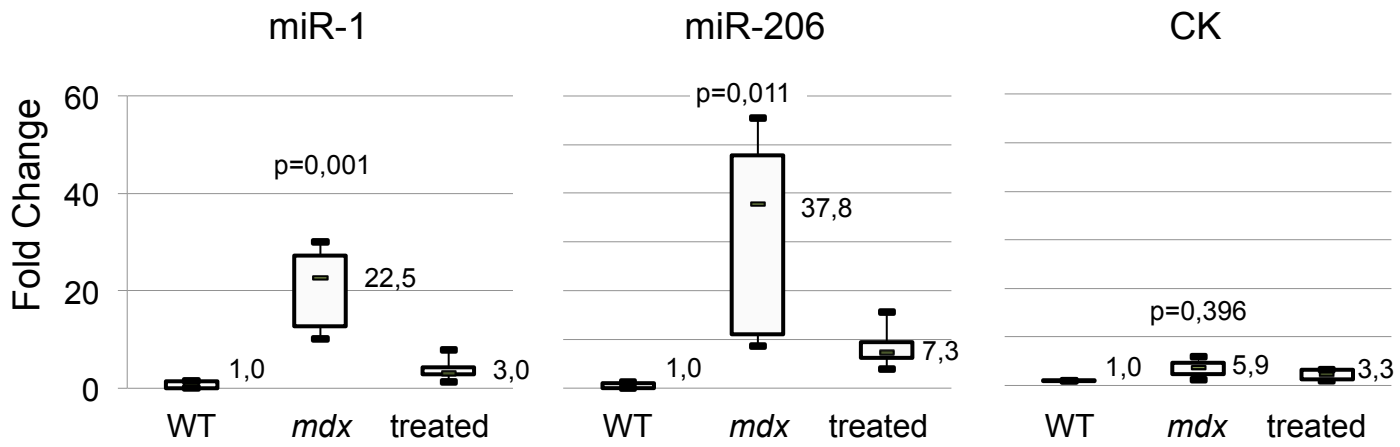






Cacchiarelli et al. 2010

miRNAs as biomarkers for therapeutic outcome measurements

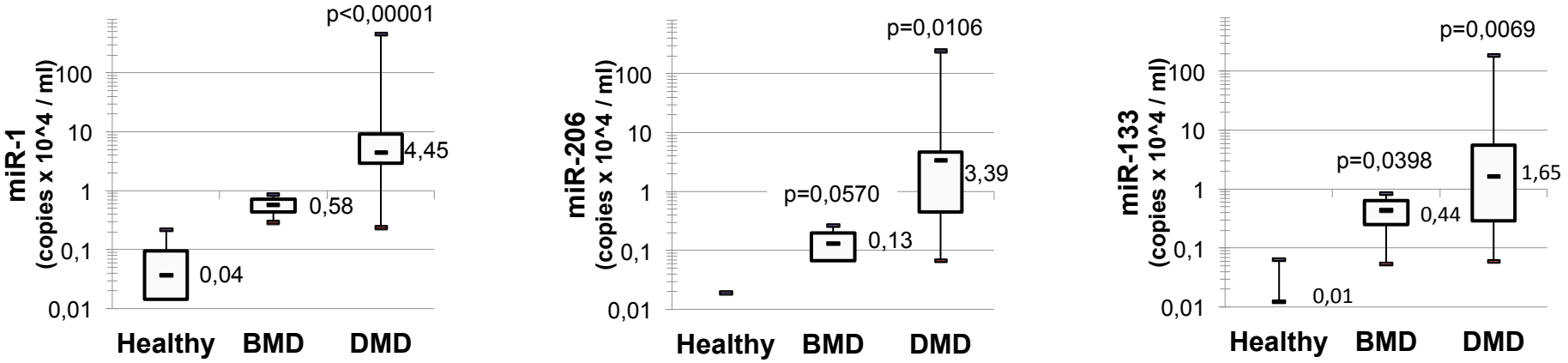


In treated mice recovery of DYS paralleled the decrease of serum muscle miRNAs to almost WT levels.

Cacchiarelli et al. 2010

Muscle-specific miRNAs as serum biomarkers for DMD

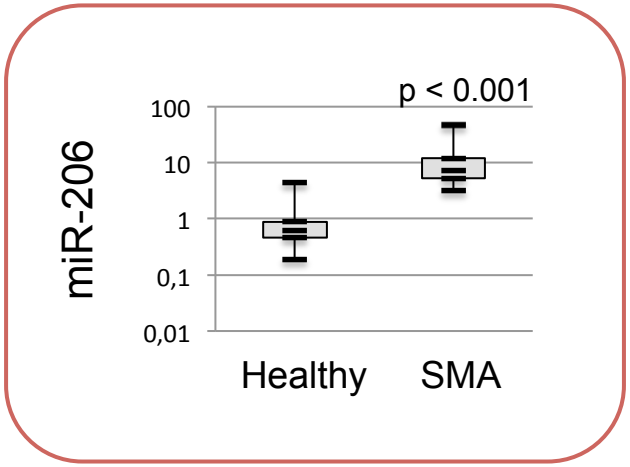
Differential amounts of muscle miRNAs in the sera of healthy versus dystrophic children

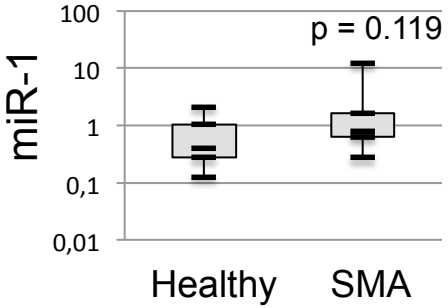


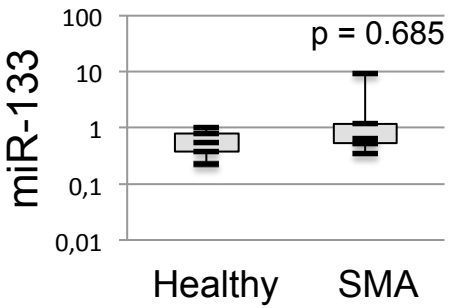
Cacchiarelli et al. 2010

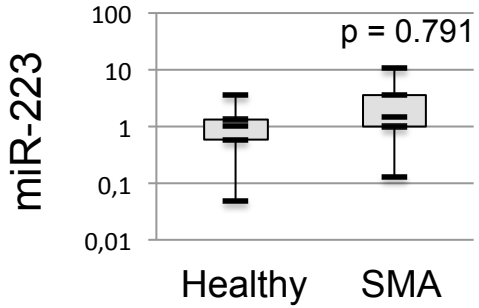
Brevetto!

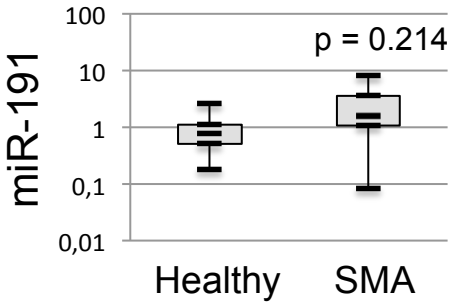
Muscle-specific miRNAs as serum biomarkers for SMA:



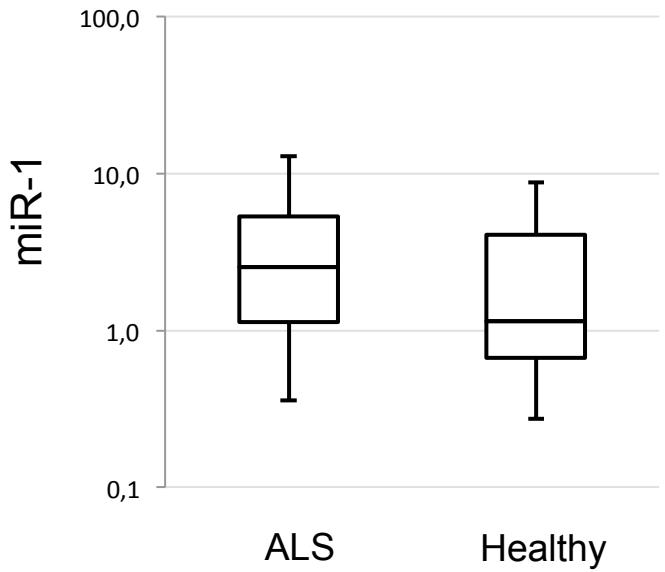


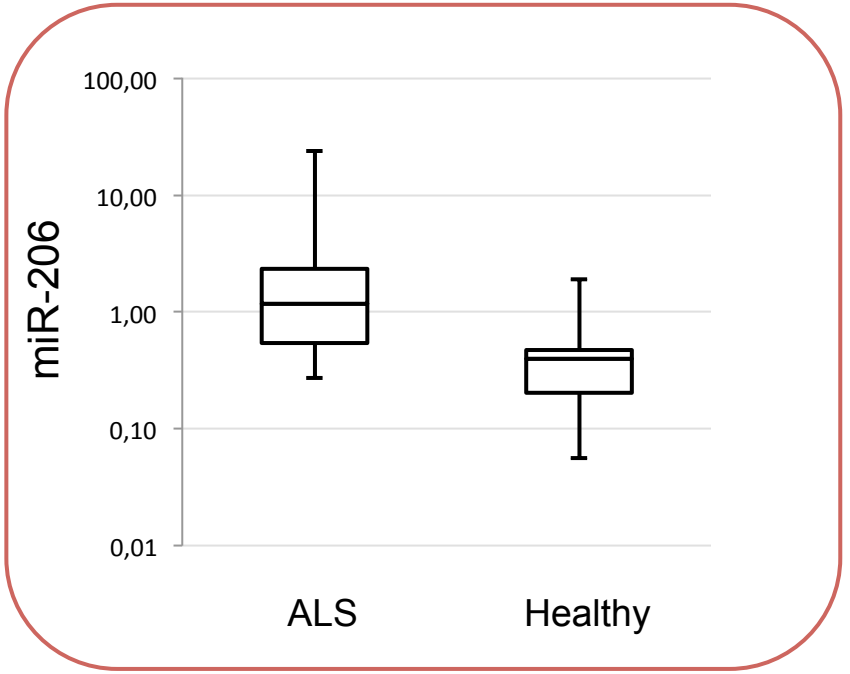






Muscle-specific miRNAs as serum biomarkers for ALS:





Type of cancer	Biomarker candidate
Prostate cancer	Fifteen serum miRNAs were over-expressed from stage 3 and 4 prostate cancer patients (miR-16, -92a, -103, -107, -197, -34b, -328, -485-3p, -486-5p, -92b, -574-3p, -636, -640, -766, -885-5p) when compared to normal individuals (Lodes et al. 2009) Expression levels of **miR-141** in serum can distinguish prostate cancer patients from healthy controls (Mitchell et al. 2008)
Breast cancer	Forty-eight serum miRNAs were differentially expressed in breast cancer patients (22 up-regulated, 26 down-regulated) when compared to controls (Zhao et al. 2010) Increased expression levels of miR-10b and -34a in serum was observed in breast cancer patients (Roth et al. 2010) Decreased expression levels of miR-195 and let-7a in serum was observed in breast cancer patients (Heneghan et al. 2010) **Circulating miR-125b expression is associated with chemotherapeutic resistance** of breast cancer (Wang et al. 2012).
Ovarian cancer	miR-21, 92, 93, 126 and 29a were over-expressed in serum samples from cancer patients compared to controls and miR-155, 127 and 99b were under-expressed (Resnick et al. 2009) Eight serum exosomal miRNAs were elevated in ovarian cancer patients: miR-21, -141, -200a, -200b, -200c, -203, -205, -214 (Taylor et al. 2008).
Oral cancer	The level of plasma miR-31 was significantly elevated in oral squamous cell carcinoma patients compared to the control groups (Czech et al. 2009). Two miRNAs (miR-125a and -200a) showed decreased levels in saliva with oral cancer (Park et al. 2009)

Type of cancer	Biomarker candidate
Colorectal cancer	diagnostici
Bladder cancer	The ratio of two urinary miRNAs (miR-126 and -182) enabled detection of urinary bladder cancer (Hanke et al. 2010)
Lung cancer	Eleven serum miRNAs (including miR-7i, -146b, -206, and -21) were changed more than five-fold by NGS between longer-survival lung cancer patient groups and shorter-survival groups. Levels of four miRNAs (miR-486, -30d, -1, -499) were associated with overall survival (Rai et al. 2005) miR-155, miR-197, and miR-182 in the plasma of lung cancer including stage I patients were significantly elevated compared with controls. The levels of **miR-155** and **miR-197** were higher in the plasma from lung cancer patients with metastasis than in those without metastasis ($P<0.05$) and were significantly **decrease**d in **responsive patients during chemotherapy** (Zkeng et al. 2011)
Papillary Thyroid Carcinoma	The expression of serum let-7e, miR-151-5p, and miR-222 was significantly increased in PTC cases relative to benign cases and healthy controls. Expression of serum **miR-151-5p and miR-222 in a subset of PTC patients decreased significantly after tumor excisio**n (Yu et al. 2012).

Pathology	Biomarker candidate
Scleroderma	The median serum levels of miR-92a, not miR-135, were significantly higher in SSc patients than normal subjects (Singh et a. 2012).
NTD fetuses	6 pregnancy-associated miRNAs (miR-142-3p, miR-144, miR-720, miR-575, miR-765 and miR-1182) that are upregulated in the serum of pregnant women with NTD fetuses (Gu et al. 2012)
Ectopic pregnancy	Pregnancy-associated **miR-323-3p**, added substantial diagnostic accuracy to a panel **including hCG and progesterone for the diagnosis of ectopic pregnancy** (Zhao et al. 2012)
Liver cirrhosis	Interestingly, miR-181b is elevated significantly in serum of liver cirrhosis cases comparing to that of normal persons, whereas miR-181a expression was in the similar level with that of normal persons (Wang et al. 2012).
Liver injury	Circulating microRNA-122 as a potential biomarker for liver injury
Heart conditions	From acute myocardial infarction patients, the level of plasma miR-208b and -499 was highly elevated and correlated with plasma troponin level (Corsten at al. 2010) Level of six plasma miRNAs includingmiR-423-5p was elevated in patients with heart failure (Tijsen et al. 2010) Plasma miR-1 level was significantly elevated from patients with acute myocardial infarction (Zhang et al. 2010; Cheng et al. 2010).

Tumor entity	References	Study Design	Sample Size	Circulating miRNAs examined	Technology	Normalization	Promising circulating miRNAs
B-Cell Lymphoma	Lawrie et al. [23]	Tumor vs. normal, retrospective study on prognosis	60 patients vs. 43 healthy controls	3	Quantitative RT-PCR	*miRNA-16*	*miRNA-155*, *miRNA-210*, and *miRNA-21*
Breast Cancer	Heneghan et al. [73]	Tumor vs. normal	83 patients vs. 44 healthy controls	7	Quantitative RT-PCR	*miRNA-16*	*miRNA-195* and *let7a*
	Zhu et al. [83]	Tumor vs. normal	13 patients vs. 8 healthy controls	3	Quantitative RT-PCR	18 s rRNA	*miRNA-155*
Colon Cancer	Huang et al. [60]	Tumor vs. normal	Screening: 20 patients vs. 20 healthy controls Validation: 80 patients, 37 adenomas and 39 healthy controls	12	Quantitative RT-PCR	*miRNA-16*	*miRNA-29* and *miRNA92a*
	Ng et al. [57]	Tumor vs. normal, tissue and serum	Screening: 5 plasma samples, associated tumor/ normal tissue 1.validation: 25 patients vs. 20 healthy controls 2. validation 180 samples	95	Quantitative RT-PCR Array	*RNU6B*	*miR-17-3p* and *miR-92*
Gastric Cancer	Tsujiura et al. [85]	Tumor vs. Normal	Screening: 8 samples and associated tissue Validation: 69 patients vs. 30 healthy controls	5	Quantitative RT-PCR	*RNU6B*	*miR-17-5p*, *miR-21*, *miR-106a*, *miR-106b* and *let-7a*
Leukemia	Tanaka et al. [56]	Tumor vs. Normal	Screening: 2 patients vs. 7 healthy controls Validation: 61 patients vs. 16 healthy controls	723	microRNA Microarray (Agilent Technologies)	*miRNA-638*	*miRNA-92a*
Lung Cancer	Chen et al. [24]	Tumor vs. normal	Screening: Pool analysis Validation: 152 patients vs. 75 healthy controls	Genome-wide profiling by Solexa sequencing	Solexa sequencing, Quantitative RT-PCR	Directly normalized to total RNA	*miRNA-25* and *miRNA-223*
	Hu et al. [74]	Study on prognosis (Overall survival)	Screening: 60 patients Validation: 243 patients	Genome-wide profiling by Solexa sequencing	Solexa sequencing, Quantitative RT-PCR	Referenced to control healthy serum sample	*miR-486*, *miR-30 d*, *miR-1* and *miR- 499*
Oral Cancer	Liu et al. [80]	Tumor vs. normal	43 patients vs. 21 healthy controls	1	Quantitative RT-PCR arrays	*miRNA-16*	*miR-31*
Ovarian Cancer	Resnick et al. [67]	Tumor vs. normal	Screening: 9 patients vs. 4 healthy controls Validation: 19 patients vs. 11 healthy controls	365	Quantitative RT-PCR arrays	*U44/U48 and miRNA-142-3p*	*miRNA-21*, *miRNA-92*, *miRNA-93*, *miRNA-126*, *miRNA-29a*, *miRNA-155*, *miRNA-127* and *miRNA-99b*
Pancreatic Cancer	Ho et al. [28]	Tumor vs. normal	Screening: 11 patients vs. 14 healthy controls, Validation: 11 patients vs. 11 healthy controls	1	Quantitative RT-PCR arrays	c. elegans spike-in *miRNA-54*	*miRNA-210*
	Wang et al. [61]	Tumor vs. normal	49 patients vs. 36 healthy controls	4	Quantitative RT-PCR arrays	*miRNA-16*	*miR-21*, *miR-210*, *miR-155*, and *miR-196a*
Prostate Cancer	Mitchell et al. [25]	Tumor vs. normal	Screening: Pool analysis Validation: 25 patients vs. 25 healthy controls	6	Quantitative RT-PCR	c. elegans spike-in *cel-miR-39*, *celmiR-54*, and *cel-miR-238*	*miRNA-141*
	Brase et al. [72]	Low grade vs. high grade	Screening: 7 high grade vs. 14 low grade Validation: 116 patients	667	Quantitative RT-PCR arrays	c. elegans spike-in *cel-miR-39*, *celmiR-54*, and *cel-miR-238*	*miRNA-141*, *miRNA-375*
Squamous Cell Carcinoma	Wong et al. [81]	Tumor vs. Normal tissue screening, Validation in serum	30 patients vs. 38 healthy controls	1	Quantitative RT-PCR arrays	*miRNA-16*	*miRNA-184*

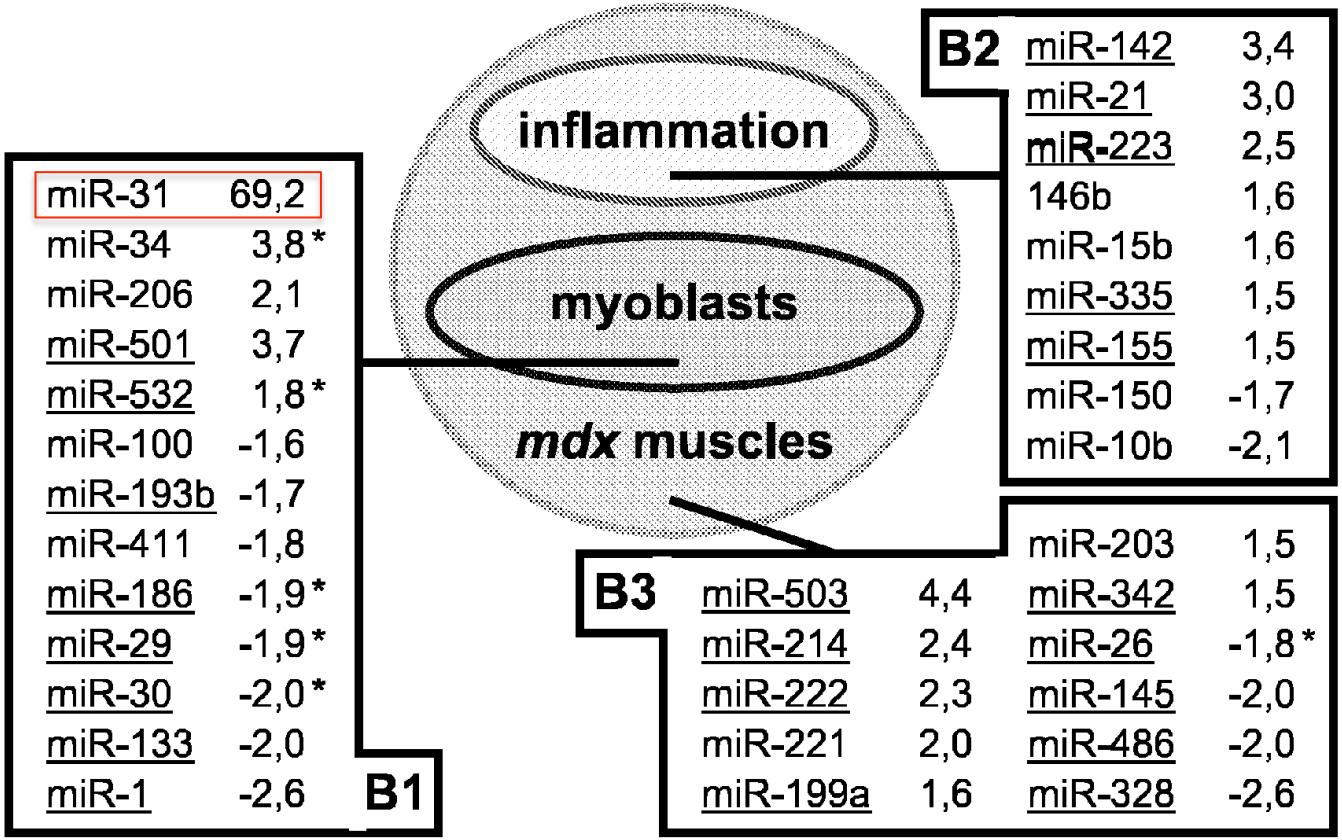
✔ Duchenne Muscular Dystrophy

✔ miRNAs and Duchenne Muscular Dystrophy

✔ Use of miRNAs as biomarkers or diagnostic

✔ microRNA therapeutics: state of the art (miRNA decoy system to enhance exon skipping efficacy)

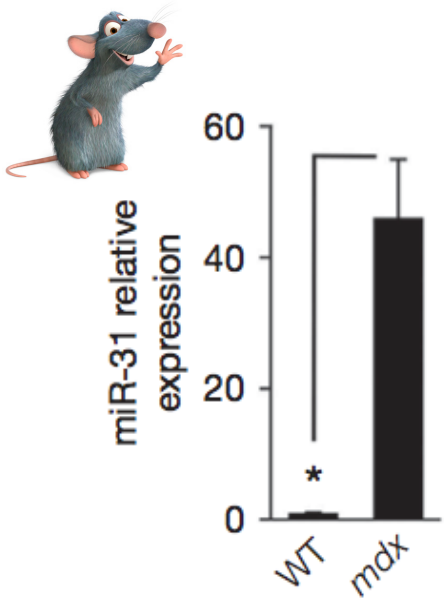
In Duchenne muscles several miRNAs are deregulated



- miR-31 is encoded by a single genomic locus and is expressed in a variety of tissues and cell types (*Landgraf P., Cell 2007)*

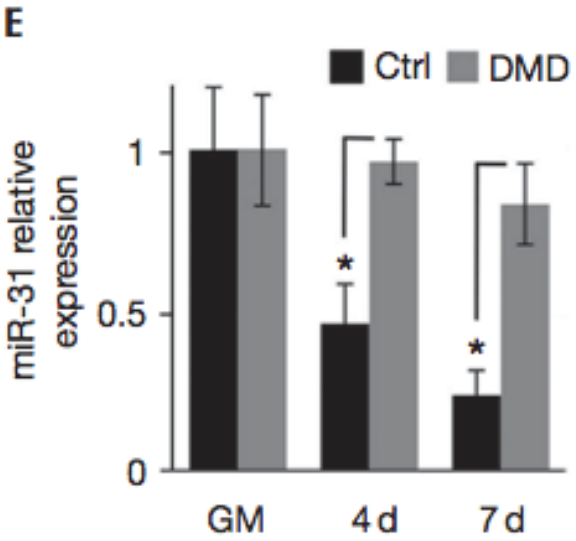
- there is an inverse relationship between miR-31 expression levels and the metastatic capacity of cancer cells: miR-31 is an inhibitor of cancer metastasis (*Valastyan S., Cell Cycle 2009)*

- miR-31 is up-regulated by more than 60-fold in the mouse model of muscular dystrophy (*mdx*) as compared to the levels of this miRNA in wild type animal. *(Cacchiarelli D,, Embo Reports, 2010)*

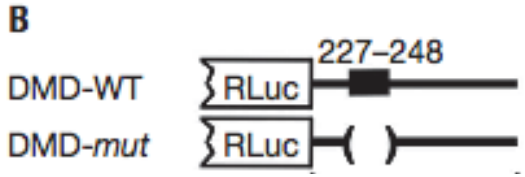


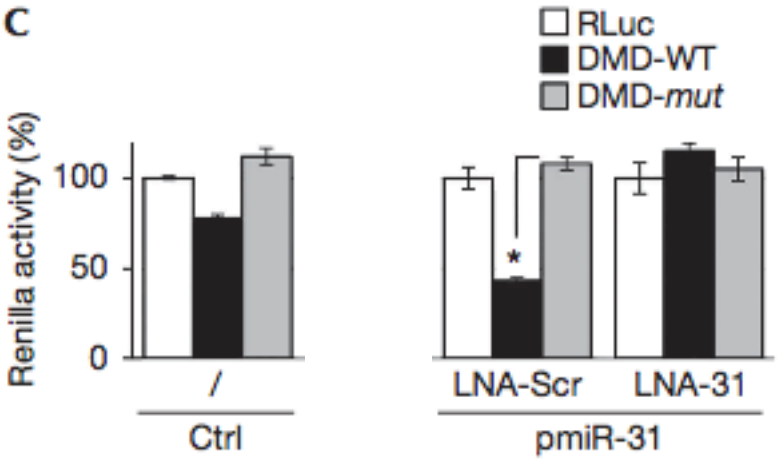
-miR-31 is up-regulated in DMD myoblasts if compared to WT myoblasts

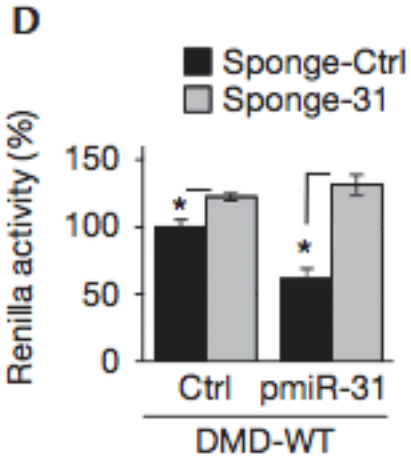
- **DMD 3' UTR presents one conserved binding site for miR-31**

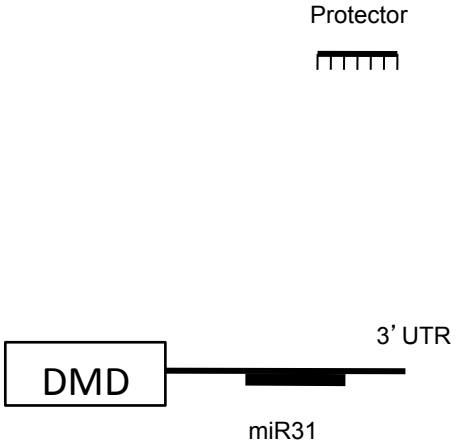


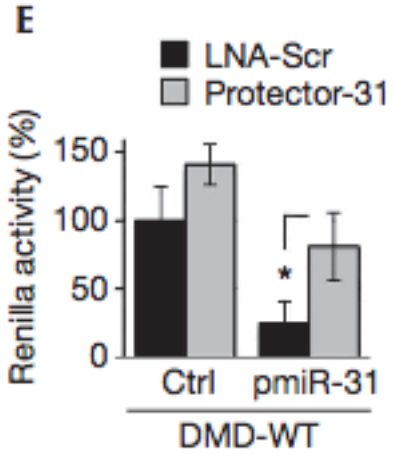
DYSTROPHIN IS TARGET OF miR-31
Luciferase assay



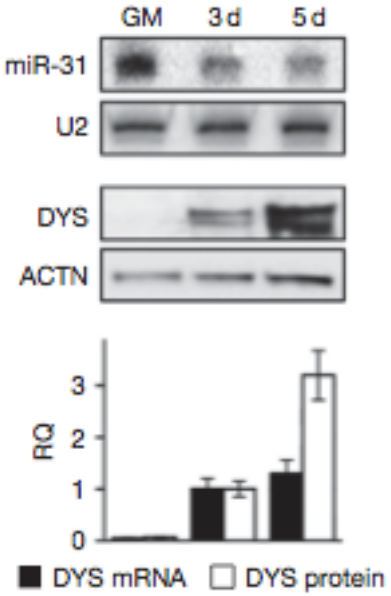






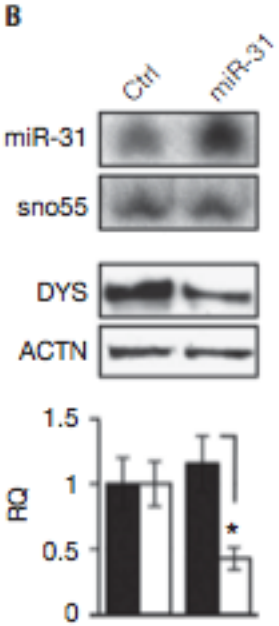


miR-31 is down-regulated during myoblast differentiation



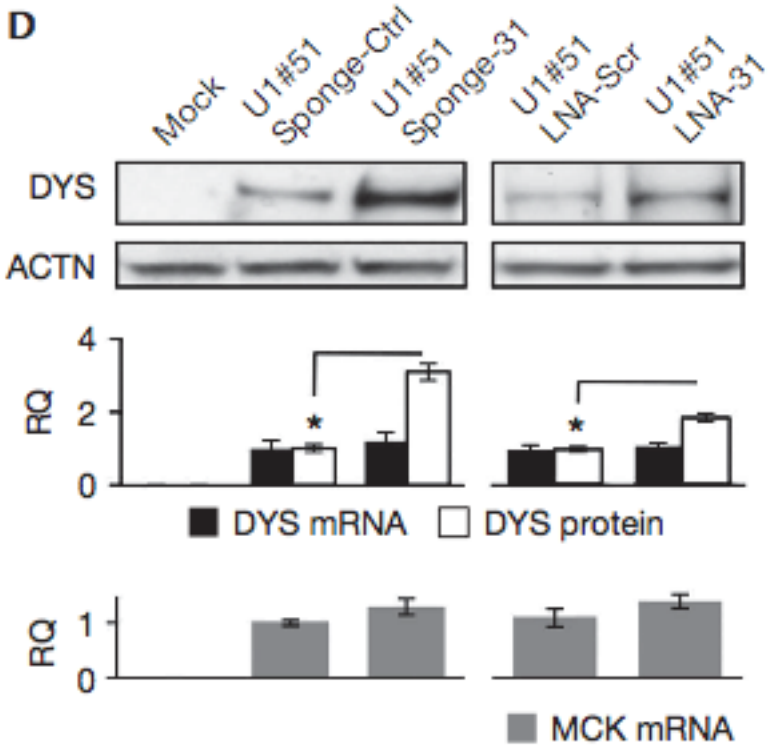
miR-31 down-regulation: dystrophin protein increases

miR-31 over-expression: dystrophin protein decreases



miR-31 and exon skipping

We can apply the exon skipping strategy to human DMD myoblast, using also a sponge construct for miR-31. This way we obtain an highr level of dystrophin if compared to the sample treated only with the exon skipping.



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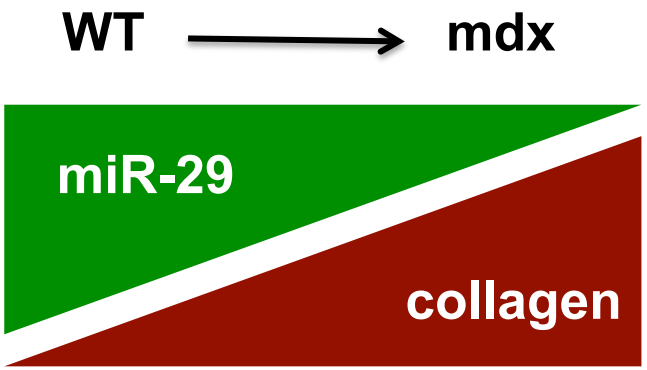
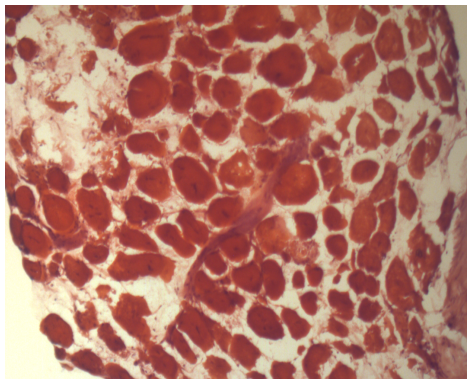
miR-29 targets proteins of the extracellular matrix which contribute to fibrosis (collagen, elastin)

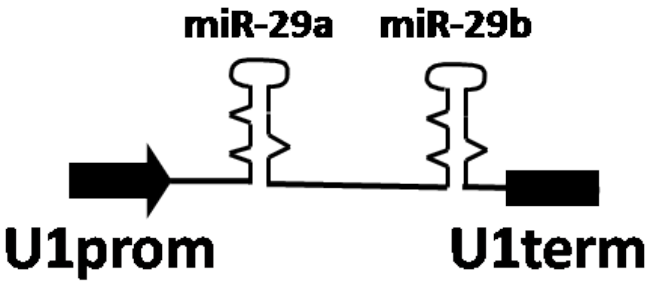


In *mdx* higher synthesis of collagen



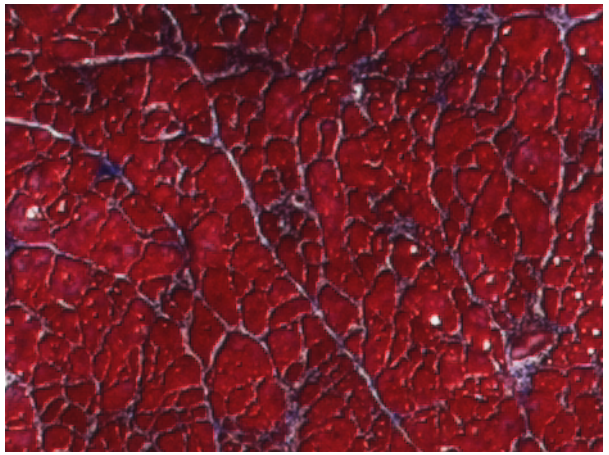
miR-29 reduces fibrosis

when administered to *mdx* muscles

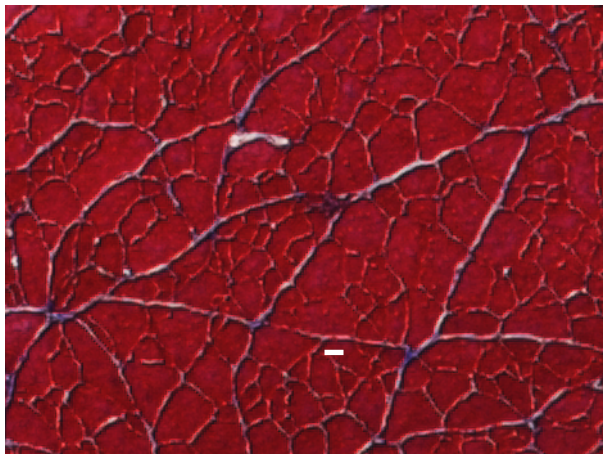


mdx

control



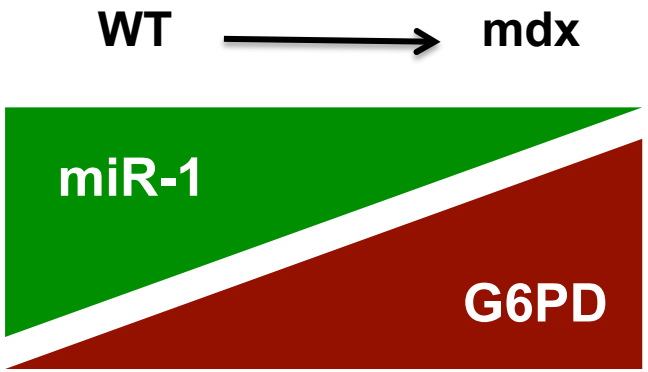
miR-29

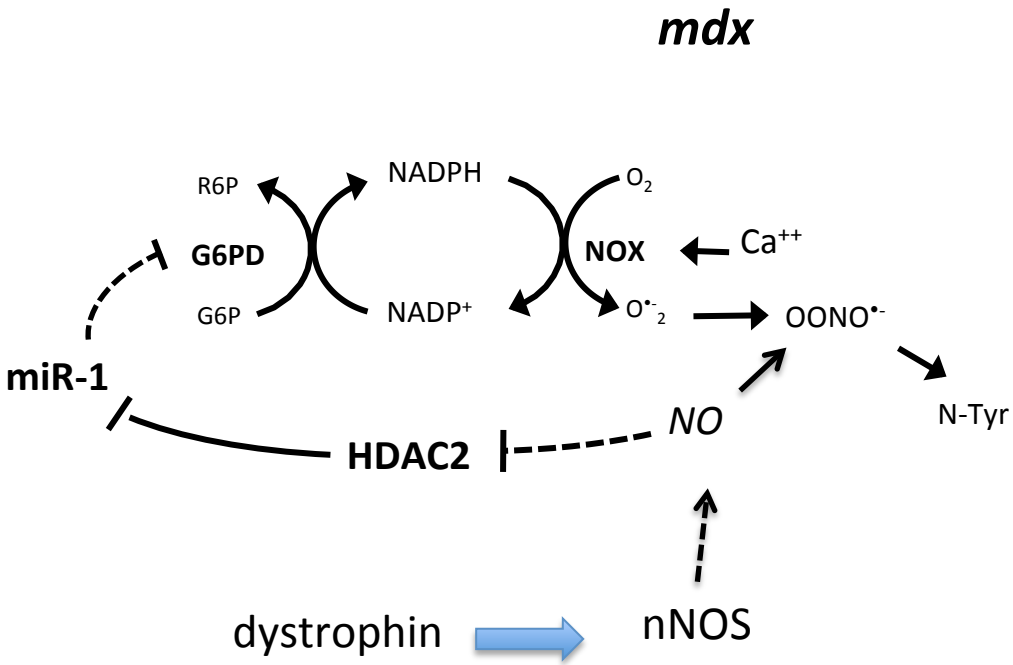


Masson's staining

miR-1 is down-regulated in mdx tissues this contributes to oxidative stress

miR-1 targets G6PD. G6PD has an aberrant action in radical production in damaged muscles





Feed forward control between G6PD and miR-1 through the S-nitrosylation of HDAC2

Detoxification of the superoxide radical could further reduce NO levels already low in *mdx*

Conclusions

- Dystrophin localizes and stabilizes nNOS

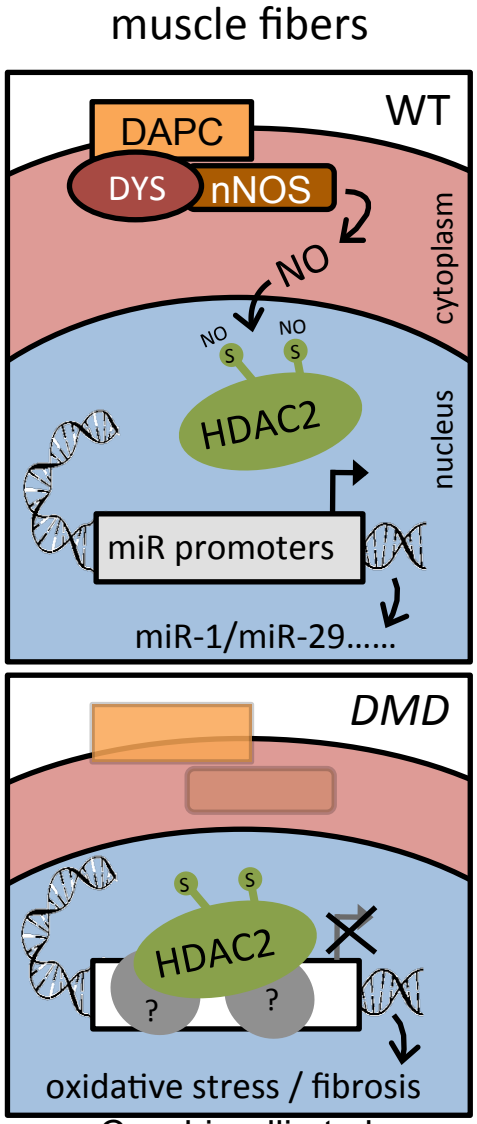
- nNOS-derived NO is responsible for HDAC2 nitrosylation

- HDAC2 nitrosylation affects its binding to miR-1 and miR-29 promoters

- miR-1 and miR-29 are key regulators of muscle physiopathology

- **dystrophin is required to increase the robustness of the muscle terminal differentiation programme**

- miR-31 and miR-206 are expressed in proliferating myoblasts before dystrophin onset – (they are not under the control of dys)



Cacchiarelli et al.
Cell Metabolism, 2010