Adeno-associated virus (AAV) are of particular interest as vectors used in gene therapy for Duchenne muscular dystrophy (DMD). In DMD, dystrophin deficiency results in secondary alterations as modifications of the microtubule network that is essential for efficient endocytic trafficking of AAV vectors. The pathophysiological muscular status should impact on crucial steps for AAV effectiveness as conformational changes which occurs in endosomal compartments, uncoating, nuclear entry and transgene expression. In this study we explored the endocytic trafficking in muscle cells of DMD patients and in mdx mice and showed that the dystrophic cellular status could affect the endocytic system and subsequently the intracellular trafficking of AAV vectors.

Endosomes, Duchenne muscular dystrophy, trafficking, AAV vector

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Gene therapies (except exon-skipping)- #3174

P10- 181- Correction of duplications in the DMD gene by a CRISPR/Cas9 approach

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Duchenne muscular dystrophy (DMD) is a severe hereditary degenerative disorder due to mutations in the dystrophin gene. The most common pathogenic changes are intragenic deletions and duplications, which account for 65% and 10-15% of all mutations in DMD, respectively. The remaining genetic causes are represented by small mutations and rarely in deep intronic variants. It has been shown that the out of frame deletion of the exon 2 of the DMD gene induce the alternative translation initiation beginning in DMD exon 6 that leads to expression of a functional N-truncated dystrophin. These results support a potential therapeutic approach for patients with mutations within the 5' exons of DMD. However, the correction of duplications in DMD and other genes require novel therapeutic strategies. Hence, genome editing technology holds great promise; by different approaches it has been demonstrated that by creating two breaks in adjacent DNA sequences, it is possible to delete the region between the two disruptions.
We report the first therapeutic approach for tandem duplications using gene editing CRISPR/Cas9 system. We believe that directing Cas9 against a region within a tandem duplications we will generate two breaks around the doubled sequence and delete it restoring the normal structure and expression of the dystrophin protein. As a model for our studies we selected the exon 2 duplication which is the most frequent duplication in the dystrophin gene.
Here we present the data regarding the design and validation of guideRNAs (gRNAs) directed against the intron 1 of the DMD gene, in a region which is duplicated in all patients with exon 2 duplication. In five out of seven gRNAs we detected cleavage activity by T7E1 assay in HEK cells.

By using combinations of these gRNAs we were able to characterize typical deletions between the target regions of two gRNAs in myogenic cell lines. Immortalized myoblasts derived from two patients with exon 2 duplication transfected with each gRNA and Cas9 resulted with a two- to four-times increase of corrected transcript in respect to untreated cells. Immunohistochemistry analysis showed rescue of dystrophin expression in several myotubes. We aim to create an mdx humanized mouse model bearing an exon 2 duplication for further studies.

The outcomes of this project, delivered in vivo, could open a novel therapeutic perspective for the correction of several severe diseases caused by this mutational mechanism.

DMD, duplications, gene editing, CRISPR/Cas9

Gene therapies (except exon-skipping)- #3205

P10- 182- Exploring CRISPR/Cas9 for therapeutic strategies for Duchenne muscular dystrophy

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Duchenne muscular dystrophy (DMD) is an X-linked recessive hereditary neuromuscular disease, affecting approximately 1:3600 boys. The disease is caused by the lack of expression in dystrophin throughout the body due to mutations disrupting the reading frame of the gene. The absence of dystrophin causes the reduction in components of the dystrophin glycoprotein complex (DGC) in the sarcolemma, leading to progressive muscle wasting, and eventually early loss of ambulation and limited life expectancy. Clustered regularly interspaced short palindromic repeat (CRISPR)/ CRISPR-associated protein 9 (Cas9) introduces a new avenue in gene therapy for DMD, and here we propose using this technology for two patients with deletion mutations of exons 48-54 and 52-54 of the dystrophin gene respectively. Both of these deletions leads to an out of frame mutation, and the removal of exon 55 in both cases is predicted to re-establish the open reading frame, yielding in a shorter but functional dystrophin protein. AAV-mediated delivery of two single guide RNAs (sgRNA) and S. aureus Cas9 components into HEK293T cells successfully removed exon 55 of the dystrophin gene. We are currently adapting and optimizing this method in patient cells, further emphasizing the potential of CRISPR/Cas9 as an individualized therapy approach for DMD patients.

P11- Gene therapies (exon-skipping) /- N° 183 to N° 188

Gene therapies (exon-skipping)- #2440

P11- 183- In silico screening based on predictive algorithms as a design tool for exon skipping oligonucleotides in Duchenne Muscular Dystrophy

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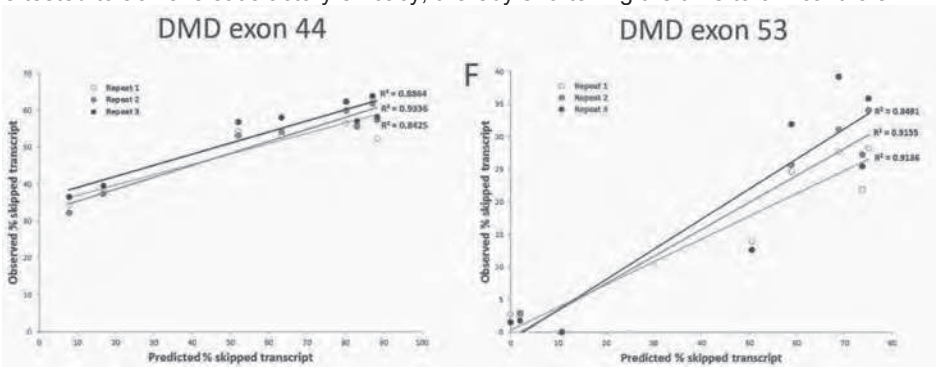
The use of antisense ?splice-switching' oligonucleotides to induce exon skipping represents a potential therapeutic approach to various human genetic diseases. It has achieved greatest maturity in exon skipping of the dystrophin transcript in Duchenne muscular dystrophy (DMD), for which a large body of data exists describing tested oligonucleotides and their efficacy. The rational design of an exon skipping oligonucleotide involves the choice of an antisense sequence, usually between 15 and 32 nucleotides, targeting the exon that is to be skipped. Although parameters describing the target site can be computationally estimated and several have been identified to correlate with efficacy, methods to predict efficacy are limited.

Here, an in silico pre-screening approach is proposed, based on predictive statistical modelling. Previous DMD data were compiled together and, for each oligonucleotide, some 60 descriptors were considered. Statistical modelling approaches were applied to derive algorithms that predict exon skipping for a given target site. In vitro validation was carried out on human-derived patient cell lines using sixteen de novo phosphorodiamidate morpholino oligomers (PMO) sequences targeting various positions on DMD exons 44 and 53.
We confirmed (1) the binding energetics of the oligonucleotide to the RNA, and (2) the distance in bases of the target site from the splice acceptor site, as the two most predictive parameters, and we included these and several other parameters (while discounting many) into an in silico screening process, based on their capacity to predict high or low efficacy in either PMOs (89% correctly predicted) and/or 2'O Methyl RNA oligonucleotides (76% correctly predicted). Predictions correlated strongly with in vitro testing of PMOs on DMD exons 44 (R2 0.89) and 53 (R2 0.89).

Our in silico screening tool can help experimenters to choose which target sites to test in vitro, and this could reduce the number of oligos that must be tested to achieve satisfactory efficacy, thereby shortening the time to clinical trials.



Exon skipping, DMD, bioinformatics

Gene therapies (exon-skipping)- #2464

P11- 184- Efficacy and toxicology evaluation of exon-skipping tricyclo-DNA antisense oligonucleotides in mdx mice

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Antisense oligonucleotides (AONs) hold promise for therapeutic splice-switching correction in many genetic diseases, and first AON-based drugs have entered clinical trials for neuromuscular disorders. However, despite advances in AON chemistry and design, systemic use of AONs is limited due to poor tissue uptake and sufficient therapeutic efficacy is still difficult to achieve. We have previously reported the therapeutic potential of 15mers-AONs made of tricyclo-DNA (tcDNA) in mouse models of Duchenne muscular dystrophy (DMD), and shown their unique pharmacological properties and unprecedented uptake in many tissues after systemic administration, including the heart and the central nervous system. Herein, we report the efficacy and toxicology profile of a 13mer-TcDNA in mdx mice. Animals were dosed once weekly for 12 weeks with 200 mg/kg per injection. We show that systemic delivery of 13mer-tcDNA allows restoration of dystrophin in skeletal muscles and to a lower extent in the brain, leading to muscle function improvement and correction of behavioural features linked to the emotional/cognitive deficiency associated with the lack of dystrophin. Remarkably in the heart, the 13mer-tcDNA induces significantly higher levels of exon skipping and dystrophin restoration than its 15mer counterpart.
TcDNA treatment was well-tolerated in all mice and findings in tcDNA-treated animals were generally limited to minimal glomerular changes and few cell necrosis in proximal tubules, with only slight variation in serum and urinary kidney toxicity biomarkers levels.

These results suggest an encouraging safety profile for tcDNA and confirm their therapeutic potential for the systemic treatment of DMD patients.

Tricyclo-DNA, antisense oligonucleotides, Duchenne muscular dystrophy, toxicology

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Gene therapies (exon-skipping)- #2476

P11- 185- Simple downstream process based on detergent treatment improves yield and in vivo transduction efficacy of adeno-associated virus vectors

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Recombinant adeno-associated viruses (rAAV) are promising candidates for gene therapy approaches. Rapid technological evolution in the last two decades led to advances in processes applied in the production and purification of rAAV resulting in better yields and higher levels of vector purity. Recently, some reports showed that rAAV produced by transient tri-transfection method can be harvested directly from supernatant, leading to easier and faster purification compared to classical virus extraction from cell pellets. We compare these approaches with new vector recovery method using small quantity of detergent at the initial clarification step to treat the whole transfected cell culture. Coupled with tangential flow filtration and iodixanol-based isopycnic density gradient, this new method significantly increases rAAV yields and conserves high vector purity. Moreover, this approach leads to the reduction of the total process cost and duration. Finally, the vectors maintain their functionality, showing unexpected higher in vitro and in vivo transduction efficacies. This new development in rAAV downstream process once more demonstrates the great capacity of these vectors to easily accommodate to large panel of methods, able to furthermore ameliorate their safety, functionality and scalability. [Published in Molecular Therapy- Methods & Clinical Development, 2015 july 15].

AAV

Gene therapies (exon-skipping)- #2477

P11- 186- Adaptive immune response impairs the efficacy of autologous transplantation of engineered stem cells in dystrophic dogs

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Duchenne muscular dystrophy (DMD) is the most common genetic muscular dystrophy, affecting 1 in 5000 male births. It is caused by mutations in the dystrophin gene, leading to absence of muscular dystrophin and a progressive degeneration of skeletal muscle and loss of function. Individuals with DMD exhibit progressive muscle weakness leading to the permanent use of a wheelchair in young adolescents, and to respiratory and heart failure in young adults. We have previously demonstrated that the exon skipping method safely and efficiently drives to the re-expression of a functional dystrophin in dystrophic CD133+ stem cells injected SCID/mdx mice. Golden Retriever dystrophic dogs (GRMD) represent the best pre-clinical model of DMD, mimicking the human pathology in many genotypic and phenotypic aspects, including the inter-individual heterogeneity. Here, we assess the capacity of serially and intra-arterially delivered autologous engineered dystrophic canine CD133+ stem cells of restoring dystrophin expression in GRMD. This is the first demonstration of five-year follow up study, showing initial clinical amelioration followed by stabilization in mild and severe affected GRMD dogs. However, the occurrence of T-cell response in three GRMD dogs, consistent with a memory response boosted by the exon skipped-dystrophin protein, suggests an adaptive immune response against dystrophin.

DMD, stem cells, GRMD

Gene therapies (exon-skipping)- #2700

P11- 187- Therapeutic exon skipping for LGMD2B

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Dysferlinopathies are a family of disabling muscular dystrophies with LGMD2B and Miyoshi myopathy as the main phenotypes. They are associated with molecular defects in DYSF, which encodes dysferlin, a key player in sarcolemmal homeostasis. Our previous investigations have suggested that exon skipping may be a promising therapy for a subset of patients with dysferlinopathies. Before further development for human treatment several important data have to be obtained using a pertinent model. We created a mouse model to test the exon skipping efficiency in a living animal. For this purpose we created a KI of exon 32 with a nonsense mutation. The mutation introduced is the c.3477C>A (p.Tyr1159X) variation identified in a patient affected with dysferlinopathy. Male mice of 3, 6, 9 and 12 months have been analyzed using standard protocol. The behavior, size of the litter, the growth curve of mice showed no significant difference. To acquire a clear picture of histological pattern different muscles were analyzed by the classical HPS staining, the cross section area the position of nuclei and the number of fibers counted. Dystrophic signs were visible as soon as 3-6 months and increase with the age of analyzed mice. The truncated protein is unstable and no protein at the expected truncated size was seen. However, RNA analysis shows that the mutated DYSF mRNA was stable. Mice were then treated for exon-skipping with 2'-O-Me-PS oligonucleotides directed against splice signal sequences of exon 32. We have done intramuscular injections in the anterior tibialis of our mouse models at the RNA level a skipped band was clearly visible. We demonstrated the presence of dysferlin also at the protein level, and we are currently testing if exon 32 skipping can rescue the mice phenotype at a functional level. Our work effort established a proof of concept for exon skipping as a therapeutic perspective for dysferlinopathies. Although some improvements may be necessary in particular at the functional level. Rapid advances obtained in clinical trials by BioMarin and Sarepta for DMD will allow a rapid translation into clinical applications in the future.

Dysferlin, Exon-Skipping, Model Mice, in vivo

Gene therapies (exon-skipping)- #2861

P11- 188- AAV genome maintenance in dystrophic muscles during AAV-U7snRNA-mediated exon skipping therapy

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Duchenne muscular dystrophy (DMD), the most common severe childhood muscular pathology, is due to the absence of the sub-sarcolemmal protein dystrophin. The dystrophin structure tolerates large internal deletions which led to the development of two main therapeutic strategies: gene therapy with transfer of micro-dystrophin cDNAs in muscles, and targeted exon skipping. Both approaches have shown encouraging results using adeno-associated viral (AAV) vectors, which allow efficient gene transfer into muscles. Exon skipping converts an out-of-frame mutation into an in-frame mutation leading to an internally deleted but partially functional quasi-dystrophin. Our team is working on the exon skipping strategy via AAVs expressing a U7 snRNA (AAV-U7). In preclinical models, a one-shot treatment of AAV-U7 was sufficient to attain substantial levels of restored quasi-dystrophin, which is associated with a significant improvement of the muscle force.

Despite the high efficiency of AAV-U7 strategy, we recently showed that quasi-dystrophin levels decreased significantly after one year in various skeletal muscles in the severely dystrophic dystrophin/utrophin knockout (dKO) mouse and GRMD dog. This decline in dystrophin was strongly correlated with viral genome loss, most likely due to alterations of the dystrophic myofiber membranes. In the context of an AAV-U7 clinical trial for DMD, AAV genome fate in dystrophic muscles is of major importance since the viral capsid immunogenicity currently limits repeated treatment. We recently investigated the viral genome fate in muscles of the moderately dystrophic mdx mouse and showed that non therapeutic viral genomes were lost quickly after the injection and that this loss was diminished when high doses of viral genomes restored the quasi-dystrophin at the sarcolemma. Our recent findings concerning AAV genome maintenance in dystrophic muscles will be presented.

Duchenne muscular dystrophy, AAV, exon skipping

P12- Metabolic and Mitochondrial Disorders- N° 189 to N° 207

Glycogenoses (except Pompe) and other metabolic myopathi- #2419

P12- 189- Grip test (non ischemic forearm exercise test) and cycle ergometer exercise comparison in McArdle disease (type V glycogenosis) and myoadenylate deaminase deficiency diagnosis.

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A retrospective study was designed to compare two exercise tests (grip test and a cycle ergometer exercise) and establish diagnostic thresholds for metabolic myopathies. 27 patients, experiencing exercise intolerance or myalgia, underwent a grip test and a cycle ergometer exercise. Simultaneously, blood lactate and ammonia were sampled. 4 patients had McArdle disease, 5 a complete MAD deficiency (MAD absent), 5 a partial MAD deficiency and were compared to 13 Controls experiencing exercise intolerance with normal muscle biopsy and acylcarnitine profile. McArdle patients showed decreased strength parameters (grip test Maximal Voluntary Contraction, p=0.038; Wmax, p=0.0025; mechanical energy, p=0.011 in grip test, p=0.006 for cycling). Work efficiency was impaired in Mc Ardle and MAD absent patients (15 and 13.1 mL O2.min-1.W-1, respectively). There was no difference between partial MAD deficiency patients and Controls. Most discriminating parameters for McArdle and MAD absent diagnosis were ammonia and lactate variations. Both tests perfectly discriminated (ROC curve AUC =1) McArdle patients, combining a low lactate variation (grip test: >1mmol/L, cycling: >0.45mmol/L) and a large ammonia variation (grip test: >100?mol/L, cycling: >20?mol/). Cycle ergometer exercise was superior to grip test to diagnose complete MAD deficiency, combining very low ammonia variation (grip test :> 10?mol/L, AUC=0.7955, p=0.066; cycling: > 8.7?mol/L, AUC=0.9045, p=0.0002) and mild lactate variation (AUC=0.7227, p=0.042 for cycling vs AUC=0.5273, p=0.79 for grip test). Grip test and cycle ergometer exercise are interesting diagnostic tools in metabolic myopathies, especially in McArdle disease, but cycle ergometer exercise is more efficient to diagnose complete MAD deficiency.

McArdle disease, type V glycogenosis, Grip test, cycle ergometer exercise, myoadenylate deaminase deficiency

Glycogenoses (except Pompe) and other metabolic myopathi- #2495

P12- 190- Characterization of a large sample of Brazilian patients with McArdle disease

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The objective was to analyze clinically, molecularly and epidemiologically 14 patients with McArdle disease (MD).Background MD is an autosomic recessive metabolical disorder characterized by onset of exercise intolerance, myalgia and painful cramping since childhood or adolescence triggered by physical activity. The incidence of MD is 1:100.000 newborn and can be attributed to mutations on PYGM gene leading to the absence of the enzyme miofosforilase b in the muscle.Methods We have studied 14 patients with MD from clinical, laboratorial and anatomical-pathological view. We have investigated the PYGM gene mutation by next generation sequencing.Results In our casuistic there are 8 men and 6 women. All of them refer symptoms beginning in