TREAT-NMD

Parent Project Muscular Dystrophy
Leading the Duchenne muscular dystrophy community

Research Approaches for a Therapy of Duchenne Muscular Dystrophy.

Updated in March and April 2008

This is a new kind of research report, which I, *Günter Scheuerbrandt*, a biochemist in Germany, have written for you, the Duchenne boys and young men and their families, who wish to know how the work of scientists and clinicians in many research laboratories of the world is progressing towards effective therapies for Duchenne muscular dystrophy. My earlier reports, and especially the last three on the Parent-Project meetings in Cincinnati in July 2006, in London 2006, and Philadelphia 2007 (www.duchenne-information.eu), contained rather detailed summaries of the research results presented at these meetings. But as also other important scientific research is performed without having yet been mentioned at the meetings, I have now shortened practically all summaries of the three last reports for this report, updated them with new information, mainly from ActionDuchenne's meeting in London in November 2007, and have added new summaries of recent important publications. References to some of the most important publications are given at the end of some summaries.

This is now a basic text, which is up-to-date as of March and April 2008. I will update it repeatedly with new information, the first time after the next Parent-Project meeting in Philadelphia in July 2008; it will be available in English, Spanish, and German a few months later. As before, all my reports and this one, too, are not scientific publications with many difficult words, because I have tried to write it in a way that will let you understand what is happening for you in the laboratories.

This report, as the earlier ones, contains summaries of only scientific research results. However, I will try to include also summaries on new medical and social management procedures in new editions of this report, based on future presentations and publications.

In the summaries, I am giving only the names of the heads of laboratories, although they have colleagues and postdocs and students working as a team on the projects reported here, but it is impossible to mention all their names. All the laboratory directors, whose work is summarized here report, have had the opportunity to see the drafts of my texts and to correct them, if necessary, and almost all have done so. Thus, there should be no, or very few, mistakes left in this report.

If you have questions concerning research, please write me an e-mail in English, German, Spanish, or Italian. I will try to answer all of them, but only in English or German.

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Introduction

How genes make proteins. **Genes** are functional units of the genetic material **deoxyribonucleic acid**, **DNA**. Its structure looks like an intertwined ladder, the *double helix*. Each rung of this ladder contains two of four different small molecules, the **bases**: *adenine*, *guanine*, *thymine*, and *cytosine* (A, G, T, C). For spatial reasons, the rungs can only contain two base combinations, the **base pairs** A-T and G-C. If,e.g., GGCTTAATCGT is the sequence of these *bases* on one strand the sequence on the opposite strand must be CCGAATTAGCA so that both sequences are *complementary* to each other:

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-GGCTTAATCGT-
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-CCGAATTAGCA-
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This sequence of the bases, of the *genetic letters*, is the **genetic information** for the development and maintenance of a living organism, and it is passed on from one generation to the next.

Most of the genes carry the instructions for the biosynthesis of **proteins**. In the cell nucleus, the genetic instruction of active genes is **expressed**, it is copied, **transcribed,** to another genetic substance, the **pre-messenger ribonucleic acid** or **pre-mRNA,** also called the *transcript*. Most genes consist of active or coding regions, the **exons**, which contain the information for the proteins, and the often much longer **introns**, which do not contain only "genetic junk", as one once thought, but also important information for the control of gene activities. After transcription, the introns are removed from the pre-messenger RNA, and the exons **spliced** together to form the **messenger RNA, mRNA**, which then moves to the **ribosomes**, the protein synthesizing

structures in the cytoplasm outside the nucleus. The ribonucleic acids, **RNA**s, use the base U, *uracil*, instead of the similar base T of the DNA. **Splice sites** are specific sequences inside the exons and at the borders of exons to introns which are essential for the correct removal of the non-coding intron sequences from the pre-mRNA. The splicing itself is accomplished by **spliceosomes**, a complex of many proteins and small RNAs.

In the messenger RNA, three consecutive bases, a **codon,** *triplet*, or *genetic word*, specify, with three exceptions, one of 20 different **amino acid**s according to the **genetic code**. There are no spaces between the codons. In the ribosomes, the genetic code words of the messenger RNA are read and *translated* into the language of the proteins, which are built of many, often thousands, of amino acids, their building blocks. The three exceptions mentioned are the triplets UAA, UAG, and UGA, which are **stop codons**, where the assembly of the protein in the ribosomes comes to a halt.

The dystrophin gene and protein. Duchenne and Becker muscular dystrophies are caused by a **mutation** or damage of the **dystrophin gene** which carries the information for the different forms of the protein **dystrophin**. With a sequence of 2,220,223 bases, it is by far the largest known human gene. Only 11,058 bases, 0.5%, in the 79 exons of the dystrophin gene specify the sequence of the 3,685 amino acids of the normal dystrophin protein in the muscle cells.

The size of the dystrophin gene and protein. The double-helix structure of the dystrophin gene is 0.75 mm long. Together with the other, at last count, 20,488 human genes, it fits into a cell nucleus of about 0.01 mm diameter only because the genetic material is extremely tightly packed. One molecule of the full-length dystrophin protein is much shorter than its gene, it is 125 nanometers (= 0.000125 mm) long, 8,000 of them laid end to end in a straight line would cover just one centimeter. And in one gram of muscle, there are 114 billion dystrophin molecules. This may help to appreciate the task of the scientists: To stop or to slow down the disease, to let the muscles function again more or less normally, at least about 30% of the normal number of the dystrophins has to appear again after the damaged gene cannot make them any more. The new ones don't have to have exactly the same form, they can be shorter, but they must be able to work properly. And that means billions and billions of new dystrophins have to come back in every gram of muscle, and a child has many kilograms of them!

The role of dystrophin. Dystrophin is needed for the mechanical stability of the muscle cells. It is located on the inside of the muscle cell membranes. One of its ends, the **C-terminal**, is bound to a group of other proteins in the membrane, the **dystrophin-glycoprotein complex,** and the other end, the **N-terminal**, connects to the contractile structures inside the muscle cells. The central portion of dystrophin, the **rod domain**, consists of twisted amino acid chains that fold back on themselves several times. If the contraction movement of the muscle cell forces the dystrophin protein to change its length, its folded structure allows it to act like a spring, like a *shock absorber*. Thus dystrophin transmits the mechanical energy produced by the actin-myosin *contraction machinery* to the muscle cell membranes and the structures outside them, the connective tissue and the tendons, in a well-balanced way that does not overstresses them.

Dystrophin has more roles: It organizes the complicated structure of the dystrophin-glycoprotein complex and the location of many other proteins. It also regulates complex processes like the maintenance of the correct amount of calcium in the cells and those controlling the growth of the muscles. Many details of these intricate interactions between numerous components in a living cell are still unknown.

Duchenne boys have no or very little dystrophin in their muscle fibers. When its protective and organizing effects are missing, the muscle contraction causes the rupture of the muscle membranes, and this allows large amounts of calcium to flow into the fibers. The excessive calcium activates enzymes like *calpain* and other proteases that break down muscle proteins and initiate cell death programs, apoptosis. The consequences are a chain of events like inflammation and activation of fibroblasts which lead to **fibrosis**, scar tissue that slows down muscle regeneration and causes the typical symptoms of older Duchenne patients.

Boys with the slower progressing *Becker muscular dystrophy* have lower than normal amounts of dystrophin that is also often shorter than normal. It still can fulfill its role, but cannot work as effectively as the normal version.

But not only the skeletal muscles suffer when dystrophin is missing, but also the smooth and heart muscles. Damage to the heart muscles produces *cardiomyopathy*, and the weakness of the smooth muscles has many consequences, among them the reduced ability of blood vessels to relax when blood flow increases leading to respiratory and other problems, and also the gastrointestinal tract is affected when the motility of the intestines is reduced. So the damage of just one gene can affect large parts of the body.

The mutations of the dystrophin gene. There are three common types of mutation that affect the function of the dystrophin gene: **Deletion**s, if one or more entire exons of the gene are missing, **duplications**, if parts of the gene are repeated, and **point mutation**s, if single base pairs are exchanged, eliminated or added. Other mutations are inversions and changes in introns that alter normal splicing patterns.

As the three-letter codons of the messenger RNA is read in the ribosomes one after the other without interruption, this normal **reading frame** is not disturbed if the mutation caused the deletion or addition of entire codons of three bases each. In this case, the reading frame remains **in-frame** and the dystrophin can still be made but it is longer or shorter than normal. If this change affects only non-essential structures of the dystrophin, it can be partly functional and thus give rise to the less severe **Becker muscular dystrophy**.

If, however, the mutation shifted the reading frame by one or two base pairs, the reading frame becomes **out-of-frame.** Then, a series of incorrect amino acids is incorporated into the protein starting at the mutation site until finally a new and **premature stop codon** is reached. The incomplete dystrophin cannot fulfill its normal function, it disappears and **Duchenne muscular dystrophy** develops.

Dystrophin in brain. Dystrophin is not only present in muscle fibers but also in other organs like the brain and the retina of the eyes. The dystrophin in the muscle with a molecular weight of 420 kd, kilodaltons (420,000 times heavier than one atom of hydrogen) is the largest of five *isoforms* (proteins of different size). In the brain, this normal dystrophin, but also the four smaller ones, are found predominantly at the synapses, where the nerve cells connect to each other, and in the walls of the blood vessels in the brain, in the so-called blood-brain barrier. That means these dystrophins are important for the communication between the nerves and also for the correct activity of the blood-brain barrier which lets only those substances pass that are necessary for the normal function of the brain.

The dystrophin gene has seven **promoters**, base sequences on which the protein biosynthesis is initiated. The first three promoters at the beginning of the gene control the synthesis of the normal protein, the other four are farther inside the gene. The closer a promoter is to the end of the gene the shorter is the dystrophin produced. This means, that mutations which have occurred before that particular promoter do not affect the production and the structure of the protein initiated by that promoter. For instance, Duchenne boys with mutations in the first half of their gene still can produce their short dystrophin isoforms for the brain in contrast to those with mutations in the latter regions of the gene, whose brain dystrophins will then also be absent. This explains why some Duchenne boys have significant learning and behavior difficulties and other boys only a few or none at all.

As the different dystrophin isoforms have also been found in the retina of the eye, the location of the mutation seems to be responsible for the color vision difficulties of some Duchenne boys.

Communicated by Prof. ***Francesco Muntoni*** of the Imperial College in London.

Exon Skipping

Exon skipping is not a cure. The *exon skipping* technique tries to slow down the fast Duchenne dystrophy into the much milder Becker dystrophy. It *does not alter the gene itself with its mutation,* but affects how the defective gene is read and processed. Exon skipping will *not* be *a complete cure for Duchenne muscular dystrophy*, it should only reduce the severity of its symptoms, it will only be *a therapy*.

If a mutation, a deletion, duplication or point mutation, disturbs the reading frame of the messenger RNA, mRNA, and thus causes Duchenne dystrophy, the frame can be *restored* by artificially removing from the mRNA one or more exons with *antisense oligoribonucleotides*, AONs. They are short pieces of RNA whose sequences are designed in such a way that they attach themselves precisely to the complementary sequence of the pre-mRNA inside the exon to be removed or at its border regions, *and nowhere else*. These AONs thus interfere with the splicing machinery so that the targeted exon or exons are no longer included in the mRNA, they are *skipped*.

As this skipped mRNA is shorter than normal, the dystrophin protein is also shorter, it contains fewer amino acids. If the missing amino acids are part of non-essential regions, like the rod domain, the shorter protein can often still perform its stabilizing role for the muscle cell membrane. The result would be the change of the severe Duchenne symptoms into the much milder symptoms of Becker muscular dystrophy.

For the first exon skipping trials, two kinds of chemically protected AONs are used. They have to be protected because then they are not or only slowly destroyed in the muscle cells by nucleic acid destroying enzymes. The two types of AONs are the *2'O-methyl-phosphorothioates*, also called *2'O-methyls* and the *morpholinos.*

Exon skipping trial in the Netherlands. The *first in-human trial with the exon skipping technique* was performed in the Netherlands between January 2006 and March 2007. It was designed to provide a *proof of principle* only and not a therapeutic benefit to the treated boys. It was a *local study* on a small area of a single muscle, the *tibialis anterior* muscle of the shin, which was treated with a 2'O-methyl antisense oligoribonucleotide, AON, against exon 51 called *PRO051*. With this type of a chemically protected AON, the Dutch researchers had worked in preclinical experiments for many years and were able to successfully skip dystrophin exons in muscle fibers not only in cell cultures but also in living mice and dogs after local and systemic injections.

Before the start of this first clinical exon-skipping trial, clinical and molecular genetic tests were performed on each boy to make sure that the exon skipping procedure in the living boys would produce the shortened Becker-type dystrophin of the expected structure. Four boys, who already were using wheelchairs, participated in this open study. They were between 10 and 13 years old and had proven deletions of the dystrophin exons 50, 52, 48-50, and 49-50. They were treated sequentially, meaning that only after the results for one boy were positive and did not show any serious side effects, the next boy was treated. Each boy received four injections of 0.2 mg PRO051 dissolved in 0.2 ml saline (0.9% NaCl) under local anesthesia in a small region of 1.5 cm length of the *tibialis anterior* muscle.

After four weeks, muscle tissue was obtained after a biopsy from the injection site and tested for the expected skipped mRNA and shortened dystrophin. These tests showed that 64%, 85%, 97%, and 73% of the muscle fibers still present in the dystrophic fibers contained new dystrophin at their membranes after this 4-week treatment.

Relative to laminin α2, a protein not affected by the dystrophic process, the dystrophin content was 33%, 35%, 17%, and 25%. This comparison takes into account the extent of the muscle degeneration. Without this adjustment the 13-year old boy with much connective tissue and fat in his muscles had only 3% of the normal amount of dystrophin, whereas the boy with the least affected muscles had 12%. Molecular sequencing methods then proved that the new dystrophin had exactly the expected structure with a restored reading frame. It was impossible to determine whether the amount of the new dystrophin would have been able to slow down the progression of the disease in the entire muscle because the treated muscle tissue volume was too small.

These results signify that an exon skipping treatment, when it becomes available, should be started when most of the muscles are still intact, that is, immediately after the precise dystrophin mutation is known which causes a shift of the reading frame.

The Dutch researchers are now preparing the next trial during which they will administer the PRO051 AON *systemically* into the blood circulation, so that it can reach all muscles including those of the lung and the heart. The injections will be done subcutaneously (under the skin) because it had been shown with mice and monkeys that this type of application caused exon skipping without serious side effects in all tested muscles, also in the heart and the diaphragm, and because it would not require frequent visits to doctor's offices and hospitals if repeated treatments will become necessary.

This exon skipping technique with the 2'O-methyl AONs was developed at the University of Leiden by Prof. ***Gertjan van Ommen***, Prof. ***Judith van Deutekom*** and their team, but for the organization and performance of the clinical trials, the company Prosensa B.V. in Leiden with its president Dr. ***Gerard Platenburg*** is now responsible. Prof. van Deutekom is now head of research of Prosensa.

The positive results of the pre-clinical studies and the local clinical trial have convinced Prosensa to start the open phase-I/II *systemic* trial in 2008 mainly to determine whether there are any side effects and some efficacy in hopefully many muscles. This study will be done 5-15-year old Duchenne boys and will last five weeks with one subcutaneous injection every week. Because it has not been proven that the AON doses used in the animal studies will be safe to use in children, too, one will begin the systemic trial with a very low dose which will be increased slowly to approach an optimal level.

Prosensa has already produced gram quantities of the AON against exon 51 in clinical grade quality for the coming trial. Also, AONs with optimized structures against exons 43, 44, 45, 46, 50, 52 and 53 have been prepared. These AONs together, including the anti-51-AON, would allow the treatment of over 65% of all patients with deletions. But, for financial reasons, Prosensa is developing at present only the AONs against exons 44 and 52 towards full clinical application and marketing. The company needs more investment capital for the development of other AONs and appreciates the substantial funding received from parents' organizations like the Dutch and German Parent Projects, the French muscular dystrophy association AFM and others.

Van Deutekom JC, Janson AA, Ginjaar IB, et al. Local dystrophin restoration with antisense oligonucleotide PRO051. N Engl J Med 2007; 357; 2677-86.

Hoffman, EP. Skipping toward personalized molecular medicine. N Engl J Med 2007; 357; 2719-22.

Exon-skipping clinical trial in the United Kingdom.

Another clinical exon-skipping trial is being performed in the UK by the *MDEX Consortium* under the direction of Prof. ***Francesco Muntoni*** of the Imperial College London. The MDEX consortium, funded by the department of Health, groups together nine researchers as well as the charities Muscular Dystrophy Campaign, ActionDuchenne, and Duchenne Parents Support Group.

Eight different antisense oligos, AONs, were tested in cultures of normal and Duchenne human muscles and in non-dystrophic mice which contained human dystrophin genes (1). The best results were obtained with the *morpholino* AON *H51A* developed by *Steve Wilton*, and shown by *Dominic Wells* to be sufficiently stable for a long-term clinical treatment. This morpholino AON is being manufactured in clinical grade by the company AVI BioPharma Inc. in Portland, Oregon, and is called AVI-4658.

Three groups of three Duchenne boys each, who are 12 to18 years old and who cannot walk anymore, are receiving three different dosages: 0.09, 0.297, and 0.9 mg morpholino AON in 0.9 ml physiological salt solution, delivered with nine injections directly into the *extensor digitorum brevis* muscles on the outside of one foot. This muscle was selected because it is easily accessible and can be biopsied without serious consequences if some unacceptable side effects should occur. The muscle of the other foot receives injections of salt solution for control tests. Extensive clinical checks including biopsies are being done before and 30 days after the injections.

After the approval by all three UK regulation authorities were given after a lengthy application process, the first boy received his AON injections on 18 December 2007. The results of the entire study are expected to be reported during 2008.

As soon as this first trial shows promising results, a systemic study, which is already in an advanced planning state, will start with subcutaneous injections of the AVI-4658 AON into the blood circulation. One of the most decisive pre-clinical animal experiments for the preparation of this trial were seven weekly AON injections into the tail vein of mdx mice which resulted in more than 50% of the normal amount of dystrophin in most of their muscles which was then present for at least 14 weeks.

In this systemic trial, it is planned to treat four groups of ambulant Duchenne boys by weekly subcutaneous systemic injections starting with a low dose of about 600 mg AON to be increased to about 3 grams, calculated for a boy of about 10 years. In previous clinical trials for other diseases, AON doses of up to 300 mg/kg/day were well tolerated, so the starting dose in this Duchenne trial is very low. The aims of the trial are to test for safety and tolerability and also to changes of muscle function and strength. And it is hoped that a lowest dose can be determined that will induce sufficient exon skipping while being well tolerated by the children without serious side effects.

Detailed description of the local clinical trial can be seen at http://clinicaltrials.gov/ct/show/NCT00159250

Arechavala-Gomeza V, Graham IR, Popplewell LJ, et al. and Muntoni F. Comparative analysis of antisense oligonucleotide sequences for targeted skipping of exon 51 during dystrophin pre-mRNA splicing in human muscle. Human Gene Therapy, 2007; 18; 798-810.

Exon Skipping with U7 gene transfer: The researchers at the Généthon Institute in Evry near Paris, Prof. ***Luis García*** and Dr. ***Aurélie Goyenvalle*** (now at the University Oxford) are trying to *combine exon skipping with gene therapy* by instructing the muscle cells to produce themselves the *antisense oligoribonucleotides*, AONs, so that they do not have to be injected repeatedly. This can be achieved by transporting into the muscles modified U7-snRNAs containing the genetic information for the construction of the AONs. *U7-snRNAs* are small nuclear RNAs which have a structure similar to splicing factors. With a second research program, the French scientists have developed a new more general exon-skipping technique that also uses the viral transfer of U7 genes.

To test the first approach, a modified gene for the U7-snRNA was constructed by adding the complementary DNA sequences for two AONs which are necessary for skipping exon 23 of mdx mice. These short snRNAs, like all other RNAs too, are also "made" by genes. This modified U7-gene, U7 SD23/BP22, together with control sequences, was inserted into AAV type 2 vectors and injected first locally into single muscles of mdx mice and then systemically into their blood circulation. This led to the appearance of dystrophin without the amino acids determined by exon 23 in up to 80% of the fibers of the treated muscles, this new and shortened dystrophin migrated to its normal position underneath the muscle cell membranes, and was stable for more than one year without causing any immune reaction. The dystrophic processes in the mdx muscles, that is, their accelerated degeneration and regeneration, were completely halted. The systemically treated mdx-mice, which were physically stressed by downhill running on a treadmill, did not develop the usual muscle damage found in non-treated mdx-mice.

This U7-gene transfer technique was then applied to treat the clinically *dystrophic* golden retriever *GRMD-dog*. These dogs have a mutation in the splice site of exon 7 which can be “repaired” by skipping exons 6 and 8. By using a modified U-7 vector containing antisense structures against exons 6, 7, and 8, shortened dystrophin at almost the normal level was obtained two months after a single local injection into one muscle. A regional systemic injection into one leg with blocked circulation resulted in large quantities of new dystrophin which was still present six months later.

In the second approach based on U7 gene transfer, the exon skipping will be mediated by a new and almost “universal” U7snRNA vector that carries a complementary DNA sequence to the exon and a free tail which has binding sites for the heterogeneous nuclear ribonucleoproteins A1/A2 (hnRNP), proteins that inhibit the splicing process for all exons. The complementary sequence of the transferred gene, once transcribed into a small RNA, will attach to the exon to be skipped, and as it brings with it the structures attracting the hnRNPs, it will induce the skipping of the exon because these proteins interfere with the splicing protein complex, the spliceosomes, sitting at the ends of this particular exon in the pre-mRNA. Thus, this kind of exon skipping is not brought about by the usual AONs but by these “universal” proteins that are the same for inhibiting the splicing of all exons. This method has already been tried successfully in the laboratory for skipping exon 51 in isolated myoblasts from Duchenne patients.

The reason for this approach is to shorten considerably the lengthy approval process possibly required for many or even all AONs used by the normal exon skipping techniques, because the new approach uses only the one “universal” tail structure in addition to the complentary DNA sequence to the sequence of the exon to be skipped.

Goyenvalle A, Vulin A, Fougerousse F, et al, and Garcia L, and Danos O. Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. Science 2004; 306; 1796-99.

Multi-exon skipping in dystrophic dogs. About 50% of all Duchenne dystrophies caused by deletions, duplications, and point mutations will need the skipping of two or more exons to restore the reading frame. Because dystrophic dogs need a double-exon skipping, experiments with them would open the way to the development of multi-exon skipping for Duchenne boys with these more difficult mutations. By theoretical considerations it was even predicted that a simultaneous skipping of the 11 exons 45 to 55 would produce a Becker dystrophy with very mild symptoms in up to 63% of Duchenne boys with deletions.

Prof. ***Terence Partridge*** of the Children's National Medical Center in Washington and his colleagues have started to develop multi-exon skipping in dystrophic golden retriever dogs, GRMD. In contrast to the mdx mice with their mild dystrophic symptoms, these dogs are physically handicapped, thus experiments with them would give results that would likely be similar to results of clinical studies with Duchenne patients. And experiments lasting several years can be performed with dogs, because they live much longer than mice.

These dogs have a mutation at the splice site of exon 7 in their dystrophin gene which causes the deletion of exon 7 in the mRNA and a reading-frame shift with a premature stop codon soon afterwards. Skipping of the two flanking exons 6 and 8 would restore the reading frame.

In a first *local* study, the researchers injected different doses of a cocktail of three morpholino AONs, two different ones against exon 6 and a third against exon 8, into the tibialis-anterior muscle of young adult GRMD dogs. Two weeks later, tissue from around the injection sites was obtained by biopsies, which then contained new and shortened dystrophin in all muscle fibers which had an almost normal-looking structure. But in addition to the two targeted exons 6 and 8, exon 9 was also skipped; however, this does not affect the reading frame. In preliminary experiments on tissue cultures, exons 6-9 were skipped in the presence of morpholinos directed against only exon 6, but this did not occur when the morpholinos were injected directly into the muscles; the cocktail of morpholinos against exons 6 and 8 were needed to skip these three exons in the

muscle of the living animal. Thus, exon skipping results in tissue culture do not reliably predict what will happen in the muscle in the living animal or person.

For a *systemic* treatment, performed in collaboration with Dr. *Shin'ichi Takeda* at the General Animal Research Facility in Tokyo, three two-month old dogs were treated by injecting the three AONs into their leg veins which also led to the skipping of the four exons 6-9. After two-month, a large percentage of the skeletal muscles had produced the predicted shortened dystrophin in a dose-dependent manner. No new dystrophin appeared in the heart muscles, because, as was known from earlier experiments, the morpholino AONs do not enter the heart.

Based on several muscle function tests, the physical state of the dogs was stabilized at the same level as it was before the treatment started while untreated dogs degenerated considerably during this time. Thus, the systemic treatment seemed to have halted their muscle degeneration. Nuclear magnetic resonance (NMR) tests were done to analyze the structure of the muscles. This non-invasive technique proved to be as informative as tests on muscle tissue from biopsies. This will be important for clinical trials with Duchenne boys because repeated biopsies could be minimized.

Thus, morpholino AONs work well in a large mammal with a similar body structure as in humans. They are not toxic, and do not cause immune rejection. However, they will have to be applied repeatedly, because their effect is not permanent, but this would allow to interrupt the treatment if problems occur. And they are only effective in tissues, such as muscle where the dystrophin gene is transcribed into pre-mRNA. The details of these very promising results will be published in the near future.

Béroud C, Tuffery-Giraud S, Matsuo M, et al. Multiexon skipping leading to an artificial DMD protein lacking amino acids from exons 45 through 55 could rescue up to 63% of patients with Duchenne muscular dystrophy. Human Mutation 2007; 28(2); 196-202.

Exon skipping with peptide nucleic acids. Two types of antisense oligoribonucleotides (AONs) for exon skipping are already used in clinical trials with Duchenne boys, the 2'O-methyl-phosphorothionates (*2'O-methyls*) in the Netherlands, and the *morpholinos* in the UK. Another group of AONs, *peptide nucleic acids* (PNAs), are now also being investigated for their exon skipping properties. Ribonucleic acids (RNAs) and peptide nucleic acids (PNAs) have different backbones: Chains of alternating phosphate and ribose units are the backbone of RNAs whereas chains of amino acids form the backbone of PNAs. Because of their non-ionic peptide bridges between the amino acid units, PNAs resemble electrically neutral proteins in their properties. They are water-soluble, very stable, can easily be modified and be designed to carry the usual bases of DNA and RNA in the correct spatial arrangement with any desired sequence, so that they can bind with high affinity to their complementary RNA or DNA base sequences. Thus, short antisense PNA chains with 20 to 30 bases are now being used for exon skipping experiments like the two other kinds of AONs.

Dr. ***Matthew Wood*** and his colleagues at the University of Oxford started to work with the antisense PNAs made of units of the most simple amino acid glycine for skipping exon 23 of mdx mice. Experiments in cell culture and by injecting them into single tibialis anterior muscles of young and old living mdx mice showed that three weeks after a single injection many muscle fibers contained new dystrophin that was stable for more than eight weeks.

To improve the delivery into the muscle tissue, the PNAs were "conjugated", attached to other peptides and proteins like arginine-rich synthetic peptides, to the transduction domain of the TAT protein of the HIV virus, and to the functional domain of the AAV shell protein. The positive exon skipping results with these PNA conjugates could even be more improved, at least in cell culture, with a novel antisense PNA whose backbone consists of alternating units of the amino acid proline another acid which also has, like proline, a five-member ring structure.

The details of these experiments cannot be reported here because they have not been published yet. They are very encouraging and may lead to antisense drugs that will be able to induce exon skipping with high efficiency.

Transfer of the dystrophin gene

Transfer of the dystrophin gene, with a virus vector. The transfer of a modified dystrophin gene with *adeno-associated viruses* (AAV) as vectors (as gene transporters) into the muscle cells is one of the strategies for a therapy of Duchenne muscular dystrophy. Successful experiments with dystrophic mice and dogs have aided in the development by the company Asklepius in Chapel Hill, North Carolina, of the *Biostrophin*™ biological nano particle, an AAV vector of serotype 2.5 for the first clinical trial of this gene therapy method.

These rather small viruses cannot be multiplied by the cells they infect because most of their genes are removed. This makes room for the coding sequences of a therapeutic gene to be transported that is not longer than about 5,000 base pairs. Therefore, the vectors used in this trial are carrying a dystrophin gene construction without the introns (a cDNA) and with parts of exon 17 and all exons 18 to 59 and 70 to 79 deleted. The transfer of such a *micro-gene* would thus not cure Duchenne muscular dystrophy but only transform it into a much slower progressing Becker form. The expected new Becker dystrophin will be about one third as long as the normal protein. In 1990, a 61-year old Becker patient was diagnosed who was still able to walk and who had this kind of shortened dystrophin in his muscles. Although the clinical result will be similar to a future exon-skipping therapy, it will not be mutation-specific: All Duchenne patients would be able to benefit from this gene-transfer method when it his fully developed.

After safety and toxicology testing of the mini-dystrophin vector in laboratory animals, a phase-Ia double-blind clinical trial with six Duchenne boys older than five years was performed in 2006 and 2007 under the direction of Prof. ***Jerry Mendell***, at the Nationwide Children's Hospi-

tal and The Ohio State University in Columbus, Ohio. The boys received the injections of Biostrophin at three sites, 0.5 cm apart, into their biceps muscle of one arm while the biceps of the other arm received only saline. Two different doses were used for each group of three patients. Samples of muscle tissue from the injection site were obtained by biopsies from four boys at four weeks and from two boys at twelve weeks after the injections. During the trial, no gene therapy-related adverse events were observed, suggesting that the procedure is well tolerated. The full results will be published as soon as all data are evaluated.

No therapeutic benefit is expected for the boys after this first trial whose main objective was to provide the *proof of principle* that this type of gene therapy does not only produce new dystrophin in a skeletal muscle of mice and dogs but in a human muscle as well without unacceptable side effects like an immunological response against the new mini-dystrophin or the vector material.

For the preparation of the next step, further animal experiments were performed with the aim to find the conditions for a first *vascular delivery clinical trial* with Duchenne boys: The vectors should only be delivered regionally into the blood circulation of the legs, because the vectors can only be produced in limited quantities and it would avoid the distribution of the viruses throughout the entire body, but would suffice to prolong ambulation. One single injection of either AAV type-6 or type-8 micro-dystrophin vectors into the temporarily blocked circulation of one hind leg of mdx mice produced micro-dystrophin in more than 80% of the fibers in the leg muscles and a significant improvement of their function for up to *one year*. These experiments were repeated with macaque monkeys whose body structure and weight are similar to small children. But these animals were not dystrophic, thus they had their own dystrophin. The vectors carried green fluorescent protein, an artificial signal protein, which is easy to analyze by its fluorescent light. The results were again very positive: 60 to 80% of the fibers in the leg muscles contained the transferred green fluorescent protein.

This promising outcome of the mice and monkey trials encouraged the researchers to prepare the next, so-called bridging or phase-Ib trial with Duchenne boys which will begin in 2008 or 2009. After this trial of the regional delivery technique, the participating boys may be able to walk longer than without the treatment and thus obtain a significant improvement of their quality of life.

Rodino-Klapac LR, Janssen ML, et al. and Mendell JJ. A translational approach for limb vascular delivery of the micro-dystrophin gene without high volume or high pressure for treatment of Duchenne muscular dystrophy. Journal of Translational Medicine, 2007;5;45.

Gene transfer with plasmids. Gene transfer with plasmids. In cooperation with the French muscular dystrophy association (AFM), the company Transgène in Strasbourg started in 1995 to test dystrophin gene transfer with plasmid vectors. This work was directed at that time by Dr. ***Serge Braun***, who is now Scientific Director of AFM. For this technique, the combined 79 DNA exons of the dystrophin gene, its cDNA, and its controlling structures were inserted into the genetic material of plasmids. Plasmids are small circular DNA structures without proteins, *naked DNA*, inside bacteria to which they mostly confer resistance against antibiotics.

After successful experiments in muscle cell cultures, with dystrophic mice and dogs, a first clinical trial started at the end of 2000 with 9 Duchenne and Becker patients, who were older than 15 years because they had to give their informed consent. The plasmid solution was injected into one single muscle of the forearm. Some new full-length dystrophin appeared in up to 25% of the muscle fibers around the injection sites. There were no signs of an immune reaction, neither against the plasmid construction, nor against the newly produced dystrophin. This phase-I trial thus showed that gene transfer with naked DNA is a safe procedure.

The French scientists then started working with the team of Dr. ***Jon Wolff*** of the company Mirus in Madison/ Wisconsin who injected similar plasmid constructions into the blood stream of single limbs of mice, rats, dogs, and monkeys *under pressure*. The pressure was produced by short-term blocking of the blood circulation of a limb with a blood pressure cuff.

As soon as sufficient plasmid vectors with dystrophin genetic material are available, a clinical trial with Duchenne patients using this new delivery system will be started, because this plasmid full-length dystrophin approach needs to be further developed for patients who are not eligible for exon skipping.

Myogenic cell transfer. Prof. ***Jacques Tremblay*** and his colleagues at Laval University in Québec City, Canada, are continuing working with the myoblast transfer technique, which is now being called *transplantation of myogenic cells*.

In a clinical trial with 9 Duchenne patients, they could show that in 8 patients up to 26% muscle fibers with new normal dystrophin were created after the injection of normal myogenic cells from a relative. The cells were injected at a distance of only 1 to 2 mm from each other into a small area of the shin muscle, the Tibialis anterior.

This type of cell transplantation would have several advantages, among them, (1) the new dystrophin would have the normal length and be under the control of its normal control sequences; (2) the positive effect would be long-term; (3) the technique could be combined with pharmacological treatments; and (4), most importantly, it could also help older Duchenne patients.

As about 100 injections per square centimeter of muscle surface are needed, the technique has first been performed on several monkeys without problems before it was used in two more than 18 years old Duchenne patients. Both patients received injections under local anesthesia into one or several entire muscles. One of these patients was 26 years old at the time of cell transplantation. Eighteen months later, 34% of the muscle fibers in a muscle biopsy were expressing dystrophin of donor origin. In this patient, the transplantation of myogenic cells permitted to double the strength of the muscle controlling the thumb (the only muscle still able to move in this patient). However, since the patient knew that he had been injected with cells in this muscle, it is possible that part of the strength increase was due to a placebo effect. The second patient was 18 year old, he received myogenic cells only in

one muscle involved in moving the wrist. He had a small increase of strength after 3 months but no increase of strength was observed after 6 months. No dystrophin positive fibers were observed in the muscle biopsy of this patient after 6 months and signs of a cellular rejection were detected. Both patients said that they would agree to receive additional injections into other muscles without hesitation.

Dr. *Tremblay* and his colleagues are now trying to block myostatin in combination with the transplantation of myogenic cells, and they are also developing a tolerigenic technique to avoid long-term use of immunosuppressive drugs.

Stem cells

Muscle regeneration with differentiating embryonic stem cells. During the development of an embryo, the precursors of skeletal muscles appear very early as *somites*, mesodermal structures on both sides of the embryonic neural tube. Under the influence of transcription factors (proteins that control gene activity – in particular Pax3), the cells of the somites differentiate (give rise to more specialized cells) to form, among other structures, the myotome which develops further to myoblasts, myotubes and finally muscle fibers. If it were possible to prepare from non-dystrophic somites those cells which are destined to become the myotome, they could be multiplied and then used to regenerate dystrophic muscle cells, because they would bring with them the intact dystrophin gene.

Prof. ***Rita Perlingeiro*** and her team at the University of Texas Southwestern Medical Center in Dallas were trying to isolate such early cells somite cells from mouse embryonic stem cells in cell cultures. They found that in order to obtain among them the myogenic (muscle forming) cells, the differentiating embryonic stem cells needed the transcription factor Pax3, whose gene they could introduce into the X-chromosome of the stem cells by genetic techniques. Using flow cytometry (a cell sorting method), the researchers could isolate myogenic cells from these Pax3-induced embryonic stem cells that had differentiated for five days. Cells that had the PDGF-alpha receptor and not the Flk-1 receptor generated a cell population that produced only muscle fibers without the risk of cancer formation.

Cells with these properties were multiplied and then injected locally into the tibialis anterior muscle and into the blood circulation of mdx mice. New dystrophin appeared in 11% to 16% of all muscle fibers and was accompanied by a significant increase in muscle force. Because, as has been shown in other gene therapy experiments with mdx mice, not all fibers in a muscle need to contain dystrophin for a significant therapeutic effect, the results of this stem cell approach may lead to an effective therapy of Duchenne dystrophy.

Darabi R, Gehlbach K, Bachoo RM, et al. and Perlingeiro RCR. Functional skeletal muscle regeneration from differentiating embryonic stem cells. Nature Medicine, 2008;14;134-143.

Muscle regeneration with muscle stem cells. Stem cells to be used for a Duchenne therapy should have the following properties: (1) They must be easy to isolate from human biological material like muscle tissue; (2) they should be easy to multiply in the laboratory to amounts necessary for a systemic treatment of children; (3) it should be possible to transfer into them "healthy" dystrophin-gene sequences with viral vectors; (4) systemic delivery into the blood circulation should be possible; (5) they have to be able to migrate from the blood circulation into the muscles; (6) they must give rise to large amounts of functional muscle cells with dystrophin and with functional satellite cells inside the dystrophic muscle tissue, and (7) they should not produce any serious side effects, especially no cancer. Two types of cells have been identified which seem to have these properties and which are adult and not embryonic stem cells: *Mesoangioblasts* and *pericytes*, which are located on the outside of small blood vessels within muscle tissue from where they can be isolated.

After preliminary positive experiments with mesoangioblasts on mice which were lacking the protein, *alpha-sarcoglycan*, Prof. ***Giulio Cossu*** and his colleagues at the Stem Cell Institute of the Hospital San Raffaele in Milan isolated similar cells called *pericytes* from the walls of capillaries (small blood vessels) inside human normal and dystrophic muscle tissue and injected them systemically into mdx mice whose immune system was inactivated. Before the dystrophic pericytes were injected, they were treated with viral vectors containing mini-dystrophin genes. In both cases, a large number of muscle fibers of the mdx mice had new dystrophin and their muscle function was significantly improved.

As the next step towards a human application, Dr. Cossu's team treated four dystrophic dogs systemically with cells from their own muscle tissue (autologous treatment) into which the gene for human micro-dystrophin was transferred, and six dogs with cells from healthy dogs (heterologous treatment) which contained normal dystrophin, but which required immunesuppression with cyclosporine. The results were much better after the heterologous treatment than after the autologous treatment. One dog which received the cells from a catheter into the aorta was walking well five months after the final of five weekly treatments. The other five dogs recovered more slowly.

These autologous experiments on dogs are now being repeated with stem cells that contain longer dystrophin gene sequences. Some control experiments are also performed to determine the effect of cyclosporin alone and to see whether the satellite cells on the new muscle fibers are also functional. After further long-term trials, again with dogs, and the preparation of the cells under clinical grade conditions, a clinical phase-I trial with Duchenne patients will be started to check for safety and appearance of at least some new dystrophin.

Muscle regeneration with genetically exon-skipped stem cells. The research teams of Prof. ***Luis García*** at the Généthon Institute in Évry near Paris and Prof. ***Yvan Torrente*** at the Stem Cell Laboratory of the University of Mi-

lan worked together for a new therapy of Duchenne dystrophy: they isolated stem cells from muscles of Duchenne patients, repaired their dystrophin gene with a genetic exon skipping method, transferred them into mdx mice where they regenerated their muscle fibers and ameliorated the dystrophic symptoms significantly.

The following is a very simplified short summary of a highly complex research strategy which needed many additional biochemical control and activity experiments, and also biological tests for muscle function.

The stem cells, mainly satellite cells, were obtained from muscle bioptic material of Duchenne boys whose dystrophin gene had a deletion of exons 49 and 50. For their experiments, the Franco-Italian researchers used only those about 1% of the cells which contained the marker protein CD133 in their membranes: These cells had been shown earlier to be able to repair muscle cells and form new ones in damaged muscle tissue. The CD133+ cells were multiplied in cell culture in the laboratory and then treated with a transforming vector of lentiviruses which carried in their genetic material genes of two antisense oligoribonucleotides for the skipping of exon 51 in a similar way as it has been done (and described here) for the genetic skipping of exon 23 in mdx mice.

Twenty-four hours before the experiments with two-month-old scid/mdx mice, which had neither dystrophin nor a functioning immune system, the animals were stressed by an extended swimming exercise to intensify their muscle degeneration-regeneration process. Twenty-four of these mice received 20,000 to 40,000 of the genetically engineered human stem cells from a Duchenne boy locally into one tibialis anterior muscle with three injections. Six other mice were treated systemically by injection of 500,000 of these stem cells into the femoral artery of one leg. At 21 and 45 days after these local and systemic injections, many tests were performed to assess the outcome of this complex genetic therapy.

The results showed a better muscle regeneration, a large amount of the expected dystrophin in the regenerated fibers without the amino acids determined by exons 49, 50 and 51, an amelioration of muscle morphology (their structure), and a significantly restored muscle function.

This technique which is related to the direct genetic exon-skipping approach as mentioned on page 5 because it uses the same U-7 system, opens the way to a new strategy for a Duchenne therapy. However, before clinical trials can be contemplated, the exact mechanism of exon skipping with lentiviral vectors will have to be understood completely, because these viruses with their charge enter the genetic material of the muscle cells in a random way, and possibly could disturb other genes or even induce tumors.

Benchaouir R, Merigalli M, Farini A, et al. and García L, Torrente Y. Restoration of human dystrophin following transplantation of exon-skipping-engineered DMD patient stem cells into dystrophic mice. Stem Cell 2007; 1; 646-657.

Pharmacological Approaches

Steroid Treatment. At the annual meeting of Action-Duchenne in London in November 2007, Prof. ***Adnan Manzur*** of the Hammersmith Hospital in London gave an overview of the present state of steroid treatment, presently the only drug treatment proven to be able to preserve or maintain the muscles of Duchenne boys for a limited time. This type of treatment is now considered the "gold standard" to which other treatments in development are compared.

Up to now, 47 clinical studies have been performed in many countries, but only six of them were scientifically important double-blind studies with fully published results. In most studies, daily application of the drugs were investigated, but some other regimes, like 10 days with followed by10 days without medication ("10 days on, 10 days off"), or medication for only 10 consecutive days each month were also tried. Some of the more important results are:

The first of the double blind studies was performed in 1991 by *Fenichel* et al. in the US with 99 boys who received 0.75 mg/kg/day prednisone for two years. The results showed for the first time in a scientifically reliable way that this treatment improved and stabilized the muscle strength of Duchenne boys for about three years. But 73% of the boys had side effects, mostly excessive weight gain.

In a double-blind study in Germany by *Reitter* et al. in 2000, the two steroid drugs prednisone and deflazacort were tested in 100 boys. Weight gain was significantly higher in the prednisone-treated boys than in the deflazacort-treated ones. But deflazacort led to many more rather benign cataracts (turbidity in the eye lenses) than prednisone. This study has not been fully published.

In 2006 *Biggar* et al. in Canada published the results of a long-term but open study with 74 boys who were 10 to 18 years old, 40 of whom were treated daily with deflazacort at 0.9 mg/kg/day for an average time of 5.5 years. The 34 non-treated boys lost their ambulation at 9.8 years, of the treated boys, 81% could still walk at 12 years, 76% at 15 years, and 30% at 18 years. Breathing and heart function remained significantly better and scoliosis (deformation of the spine) developed less often in the treated boys.

King et al. in the US reviewed 2007 the clinical history of 143 boys, 75 of whom were treated daily with mainly deflazacort for an average of 8 years, and 68 were not treated. The treated boys could walk 3.3 years longer and had a significantly decreased risk of scoliosis than the untreated ones. But they had an increased risk of vertebral and lower limb fractures due to osteoporosis compared with the untreated boys.

Dr. Manzur concluded his review with the following take-home message: The long-term use of the steroids prednisone (and the very similar prednisolone) and deflazacort improve muscle strength and function and prolong walking for several years, improve breathing and heart function, lower the risk of scoliosis and enhance the quality of life. The positive effects appear to be more pronounced if the treatment is started early, at about four years, and if the drugs are given daily. The side effects, especially of prednisone, are increased appetite which may lead to excessive weight gain and cushingoid face (moon-faced) unless a diet rich in proteins and low in fat and car-

bohydrates is strictly followed from the beginning of the treatment. Both drugs lead to reduced growth and increased risk of bone fractures, and to some behavioral changes. Deflazacort gives rise to some rather benign cataracts which need not to be treated.

As further research is needed, the *North Star Clinical Network*, a collaboration of 15 centers in the UK with the British parents' organizations Muscular Dystrophy Campaign and ActionDuchenne, is planning to optimize and standardize the steroid therapy of ambulant Duchenne boys by creating a national clinical database, and to offer daily and intermittent steroid treatment to all Duchenne boys from age four years onwards, or even earlier, with scientific and clinical checks and investigations before, during and after the treatment.

TREAT-NMD, the European neuromuscular network, has published 8 pages of preliminary recommendations for standards of care in Duchenne muscular dystrophy, among them about the use of corticosteroids. This text can be seen and downloaded from the internet at www.treat-nmd.eu/soc/eng/dmd/.

Fenichel GM, Florence JM, Pestronk A, Mendell JR, et al. Long-term benefit from prednisone therapy in Duchenne muscular dystrophy. Neurology 1991;12; 1874-1877.

Biggar WD, Harris VA, Eliasoph L, Alman B. Long-term benefits of deflazacort treatment for boys with Duchenne muscular dystrophy in their second decade. Neuromuscular Disorders 2006; 16; 249-255.

King WM, Ruttencutter R, Nagaraja HN, et al. Orthopedic outcome of long-term daily corticoid treatment in Duchenne muscular dystrophy. Neurology 2007; 68; 1607-1613.

Clinical trial with prednisone and cyclosporin: A clinical trial with prednisone and cyclosporin is being performed in Germany under the direction of Prof. ***Rudolf Korinthenberg*** of the Children's Hospital of the University of Freiburg with the aim to possibly reduce the side effects caused by prednisone alone. Cyclosporin is a drug which reduces immune reactions.

This trial has started in 15 German clinical centers in 2004 in which one half of the patients receives 3.5 – 4 mg/kg/day cyclosporin combined with 0.75 mg/kg/day prednisone and the other half the same prednisone dose alone with a placebo instead of cyclosporin. Each patient is treated for 15 months. In order to evaluate the study correctly, at least 150 patients are needed. They have all been enrolled and most of them have now completed their treatment.

Because of the double-blind design, no results about the combined effects of cyclosporin and prednisone will be available before the data are fully analyzed. However, the study was functioning very well and no severe side affects have appeared. The publication of the results is expected at the end of 2008.

Upregulation of utrophin to replace dystrophin. Utrophin is a protein with a structure and function very similar to dystrophin. In humans, its gene is located on chromosome 6, has 75 exons, and is about one million base pairs long. Utrophin is present in many body tissues, also in muscle, but there it is concentrated at the *neuromuscular junctions,* where the motor nerves contact the muscle membranes. At 12 weeks during the fetal development of a child, the muscle membranes contain both, utrophin and dystrophin, and then utrophin disappears until at birth only dystrophin alone remains there. Thus utrophin is a fetal form of dystrophin.

Mdx mice whose utrophin gene was *knocked out* experimentally, which have neither dystrophin nor utrophin in their muscles, have Duchenne-like symptoms in contrast to "normal" mdx mice whose muscles show less severe damage in spite of the absence of dystrophin. By increasing the amount of utrophin in mdx mice three to four-fold with genetic techniques, which cannot be used in humans, the development of the rather light dystrophic symptoms of the mdx mice could be prevented. In Duchenne patients, utrophin starts to spread from the nerve-muscle junctions to the muscle membranes, and the more utrophin a patient has, the later he must use a wheelchair. This means, that the *upregulation* of the utrophin gene would lead to a treatment for Duchenne dystrophy.

Utrophin exists in two similar forms, but only the *A-utrophin* is exclusively located in rather small amounts at the neuromuscular junctions of all muscle cells. The researchers started to look for substances that could upregulate the gene for A-utrophin and then direct this protein to the muscle cell membranes where it would occupy the sites vacated by dystrophin in Duchenne boys.

This research and development work was started by Prof. Dame ***Kay Davies*** of the University of Oxford, and is now being done by the company *Summit plc* near Oxford under the direction of Dr. ***Jon Tinsley.*** At the end of 2007, over 30,000 chemical compounds have been screened for their ability to upregulate the activity of the utrophin gene in tissue cultures from mdx mice. A number of active compounds were identified and the most promising series is now being optimized and tested in living mdx mice with the aim to increase the amount of A-utrophin sufficiently in all muscles of the animals.

Additional screening tests with dystrophic zebrafish are ongoing which will possibly identify other pharmacological drugs for treating Duchenne dystrophy. Zebrafish embryos are very small (2-3 mm), transparent and are fully developed by 24 hours. The muscle structure can be easily seen and analyzed under the microscope when viewed under polarized light. Muscle pathology (muscle structure) of the embryos without dystrophin is very similar to Duchenne muscle.

After further optimization, one of the most active compounds, SMT C1100, led to recovered muscle function in mdx mice, because their degeneration, fibrosis, fat deposition, and chronic inflammation was reduced significantly. After daily injections for 28 days, no side effects appeared. If ongoing preclinical toxicology and manufacture continues to be successful, safety trials with healthy volunteers could begin in 2008, followed by clinical trials with Duchenne patients in 2009.

Utrophin upregulation with biglycan. During development, the protein *biglycan* is present at the outside of the skeletal and heart muscles and connects with its two ends the proteins alpha- and gamma-sarcoglycan, which are two

components of the dystrophin-protein complex in the muscle cell membranes. Biglycan is important for the regulation of many signaling and structural proteins of the muscle membrane. Experiments done by Prof. ***Justin Fallon*** and his co-workers at Brown University in Providence, Rhode Island., with non-dystrophic mice, whose gene for biglycan had been deactivated, showed that in the absence of biglycan many proteins of the dystrophin complex had disappeared. Treating these mice with local and systemic injections of recombinant (artificially made) human biglycan led to the re-appearance of the protein beta-syntrophin and alpha-dystrobrevin, which was an indication that the dystrophin complex had been restored. The most surprising finding was that two to three weeks after systemic single injections of human biglycan into mdx mice, their normally low level of utrophin was upregulated about 2.5 fold. After three months of repeated systemic injections, the muscles of these mice without dystrophin were much more resistant to damage caused by forced lengthening and contractions.

As the two proteins to which biglycan binds, are only present in skeletal and cardiac muscles, biglycan could be active primarily in these two types of muscles, and thus may have minimal side effects. Immune reaction is not expected to be a problem because biglycan is present during development in humans. Since it acts outside the muscle cells, biglycan does not have to cross the muscle membranes when used as a therapeutic agent.

Experiments with animals will continue to optimize treatment conditions. And after sufficient human biglycan in clinical grade purity is available, a phase-I clinical trial could be started in about two years.

Transfer of the utrophin gene. Prof. ***George Karpati*** and his co-workers at McGill University in Montreal, Canada, transferred the entire gene of utrophin with a single injection of an adenovirus vector system into the tibialis anterior muscle of newborn and adult mdx mice. Afterwards, 58% of the fibers of the injected muscle in the newborn and 35% in the adult mice contained utrophin in the places underneath the membranes normally occupied by dystrophin in healthy mice. The proteins of the dystrophin-associated complex were restored for up to one year.

The new utrophin at the cell membranes prevented the necrosis (the dystrophic damage) of the injected muscle in the newborn and stopped it in the adult mdx mice. Physiological tests showed that the function of the entire treated muscle was improved. As utrophin is normally present at the nerve-muscle junctions, no immune responses were produced against the new utrophin.

However, the increased amount of utrophin in the adult mice, but not in the newborn mice, decreased with time. This is an indication that such a genetic treatment, if it could be successfully repeated in children, should be applied as early as possible in Duchenne patients.

Deal JR, Danialou G, Larochelle N, et al., and Karpati G. Successful compensation for dystrophin deficiency by a helper-dependent adenovirus expressing full-length utrophin. Molecular Therapy 2007; 15; 1767-74.

Reading through premature stop codons with PTC124. In about 13 to 15% of all Duchenne patients, the disease is due to a nonsense mutation in the dystrophin gene. This type of mutation is a single-point change that results in the introduction of a premature stop codon into the dystrophin mRNA. Such a premature stop codon causes the protein synthesis to shut down prematurely before the new dystrophin is fully assembled. The incomplete dystrophin is too short to fulfil its normal function, it is destroyed, and Duchenne muscular dystrophy develops.

PTC Therapeutics, Inc, a company in South Plainfield, New Jersey, has – under the direction of Dr. ***Langdon Miller*** – developed a drug, *PTC124*, which allows the protein-making system in the cell to *read through* such a premature stop codon in the mRNA, so that the full-length protein can be made. Such a treatment is different from gene therapy or exon skipping. To decide whether a boy with Duchenne muscular dystrophy boy can benefit from PTC124, the presence of a premature stop mutation must be proven by genetic analysis.

Details about this new drug, including its molecular structure, have been published in Nature in May 2007 with a commentary. PTC124 is a white crystalline powder which can be taken by mouth after mixing it with water, or milk. PTC124 was discovered using an automated screening program in which about 800,000 compounds of low molecular weight were tested for their read-through ability. One of the most effective among the active compounds, PTC124, was optimized chemically and then extensively tested in the laboratory. In pre-clinical experiments in muscle cultures, dystrophin was produced. In mdx mice, which have a premature stop codon in exon 23 of their dystrophin gene, it was shown that PTC124 induces full-length dystrophin production, resulting in reduced injury during muscle contraction, and decreases the creatine kinase (CK) activity in the blood. Thus, PTC124 may help the muscle cells to overcome one of the genetic causes of Duchenne muscular dystrophy.

PTC124 does not read through normal stop codons which have a different structural environment compared to premature stop codons. Toxicity studies in mice, rats, and dogs with high doses of the drug have shown an acceptable profile for continuing clinical development of the drug.

A phase-I clinical trial of PTC124 was performed in 61 healthy 18- to 30-year old adult volunteers who received the drug 3 times per day for 2 weeks. With this treatment, a serum concentration of 2 to 10 micrograms/ml could be maintained that was known to be active in mdx mice. Doses of up to 100 mg/kg/day were well tolerated by these healthy adults without serious side effects. This dose is larger than that planned to be given to Duchenne boys.

These results allowed the start of a phase-IIa clinical trial with Duchenne boys, which was performed between December 2005 and May 2007, and in which 38 boys, 5 to 17 years old, participated. They were a representative group of patients, 33 could still walk, 29 received steroids, 26 had the stop codon UGA, 6 UAG, and 6 UAA between the exons 6 to 70. The trial was not designed to produce any therapeutic benefit. Six boys received 16 mg/kg/day PTC, 20 boys 40 mg/kg/day, and 12 boys 80 mg/kg/day divided into 3 portions per day. The patients were clinically evaluated for up to 21 days before the treatment, then received the drug for 28 days, and finally had follow-up examinations again for 28 days. Muscle biopsies were per-

formed before and after the treatment on the *extensor digitorum brevis* (EDB) foot muscle to check for the restoration of full-length dystrophin production. Before the treatment, muscle tissue from the first biopsies was treated with PTC124 in the laboratory. The expected dose-dependent increase of full-length dystrophin was detected in the tissues from all boys.

The expected dose-dependent increase of full-length dystrophin was detected in the muscle tissues from all boys when tested in the laboratory. The analyses of the muscle tissue from the biopsies after the treatment detected in 19 of the 38 boys, with qualitative increases of new dystrophin expressed at low levels. The main reasons why new dystrophin was not found in all boys and not in larger amounts could be that the treatment period was too short and that the EDB muscle was probably not the best one to be analyzed because it has such a low rate of degeneration and regeneration. However, all boys showed a reduction of the blood CK level during treatment. Blood CK increased again after the treatment as expected for a drug that has to be taken continuously. Some parents and teachers observed that 2 to 4 weeks after the rather short treatment, the boys showed greater activity, increased endurance, and less fatigue than before the treatment. While these anecdotal results must be considered cautiously, the time course of symptomatic changes suggested a drug effect. Some mild to moderate adverse effects were observed but these were not clearly caused by PTC124 and were not clinically relevant.

To understand the long-term risk and benefits of PTC124, a randomized controlled long-term phase-IIb clinical trial is now being started. This trial will enrol 165 patients who are at least 5 years old and still ambulatory (able to walk ≥75 meters). Boys who are on corticosteroids will be allowed to continue that treatment. Participants will be randomised to one of 3 study groups: higher-dose PTC 124, lower-dose PTC124, or placebo. Treatment will continue for 48 weeks. The primary outcome measurement will be the distance the boys can walk in 6 minutes, comparing the results before the treatment with the same measurement during the treatment. There will be 10 additional secondary outcome measurements. After completion of the trial, all patients, including those who were on placebo, will receive long-term therapy with the higher dose of PTC124.

For this trial, an international steering committee has been established which will organize and oversee the collaboration of many clinical centers in Europe, Australia, Israel, Canada and in the United States. Drs. *Kate Bushby* and *Thomas Voit* are the European experts in Duchenne muscular dystrophy who are participating in the steering committee. The trial is ongoing in the US and will soon be opened in other countries. If this large phase-IIb trial shows good therapeutic effects, marketing approval will be sought from the regulatory agencies FDA in the United States and EMEA in Europe.

Welch EM, Barton ER, Zhuo J. PTC124 targets genetic disorders caused by nonsense mutations. Nature 2007; 447;87-91.

Schmitz A, Famulok M. Ignore the nonsense. Nature 2007;447;42-3.

Project Catalyst is a program of PTC Therapeutics for finding and developing small chemical compounds as drugs for a therapy of Duchenne muscular dystrophy.

Project Catalyst, directed by Dr. ***Ellen Welch***, was started in May 2004 to identify with automatic screening methods among several hundred thousand compounds those that could up- or downregulate the production, the *expression*, of four biological targets in muscle cells and thus maintain and improve muscle structure and function in Duchenne patients. The downregulation of *myostatin* and the upregulation of the muscle specific *insulin-like growth factor* IGF-1 would promote muscle growth and regeneration. The upregulation of *utrophin* and *alpha7-integrin* would stabilize the muscle membrane and thus improve muscle function.

The automatic screening methods for finding these potential Duchenne drugs use a newly developed test procedure which measures the light intensity of a reporter-protein, the enzyme luciferase from fireflies. A small number of compounds with at least some of the desired properties have now been optimized in the laboratory. In addition, work was initiated on another protein target, the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2a) to help maintain proper contractile function of the heart. These very promising potential drugs will be further optimized so that phase-I clinical studies with Duchenne boys could start in the near future.

Inhibition of myostatin: *Myostatin* is produced in muscle cells as an inactive protein consisting of 375 amino acids. It circulates in the bloodstream as an inactive molecule. When it's so-called *propeptide* is degraded, myostatin is activated and binds to a receptor protein, called *activin type II*, in the muscle cell membrane. The receptor-bound myostatin initiates a chain (a cascade) of chemical reactions that reaches the muscle cell nucleus and blocks muscle-forming genes. Thus, myostatin *limits* the growth of muscles.

There are cattle, the Belgian Blue Breed, and dogs, bully whippets, which are very muscular because their myostatin gene is inactivated by a mutation. And in Berlin, a physically very strong boy without myostatin was identified in 1999, whose skeletal muscles are about twice as large as those of a normal child.

Stopping myostatin with an antibody. Adult mdx mice with an inactivated myostatin gene, which, in addition of not having dystrophin also could not make any myostatin, had more normal muscles fibers, less fibrosis (scar tissue), and regenerated their muscles faster than "normal" mdx mice, as shown by Prof. ***Kathryn Wagner*** of the Wellstone Muscular Dystrophy Center at the Johns Hopkins University in Baltimore and her research team. This and the above-mentioned cases indicate that the downregulation or inhibition of myostatin would stimulate the regeneration of the muscle fibers of Duchenne boys so that they would not degenerate as fast or might even increase in size.

The company *Wyeth Pharmaceuticals* in Collegeville near Philadelphia in cooperation with Dr. Wagner recently published a phase-I/II clinical trial with *Myo 029*, a specific *human antibody* against myostatin which is injected systemically. The trial was conducted in 116 adults with

muscular dystrophy including Becker patients who received intravenous MYO-029 in four doses between 1 and 30 mg/kg every two weeks for 24 weeks, followed by 12 weeks of clinical supervision. The aim of this phase-I/II clinical trial was to assess safety and to prove some efficacy. The results showed that MYO-029 was safe at the doses studied and likely reached its intended target with increases in muscle mass in the Becker population. However, improvement in muscle function was not shown in this six-month study. Further clinical trials with other myostatin inhibitors are planned by several pharmaceutical companies in the U.S.

Wagner KR, Fleckenstein JL, Amato AA, et al. and Mendell JR. A phase I/IItrial of MYO-029 in adult subjects with muscular dystrophy. Ann. Neurology 2008; March 11

Stopping myostatin with its propeptide. Prof. ***Keith Foster*** at the School of Biological Sciences of the University of London and his colleagues used a stabilized propeptide to bind myostatin and thus to inactivate it. They transferred the propeptide with plasmids (naked DNA) locally into a single muscle of non-dystrophic mice, or systemically with an AAV8 vector into their blood circulation. In both cases, after ten weeks, the size of the muscle fibers and the muscle function increased by about 20 – 30% predominantly in slow muscles. However, the same treatment of mdx mice did not lead to increased muscles and better function. A simultaneous viral transfer of propeptide and micro-dystrophin did not show any benefit either in mdx mice. But this combination might be a future therapeutic strategy for producing new fibers and increasing them at the same time. Mdx mice probably need higher doses, because they produce more myostatin than non-dystrophic mice. This is presently being investigated.

Increasing muscle mass and strength by stopping myostatin with follistatin. *Follistatin-344* is a hormone-like protein consisting of a chain of 344 amino acids which is activated to follistatin-315 by splitting off 29 amino acids from its carboxyl end. Follistatin-315 together with two other similar proteins, follistatin-related protein (FLRG) and the growth and differentiation factor-associated serum protein-1 (GASP-1) are involved in regulating the activity of myostatin directly by blocking activin, its receptor, and indirectly also by other, still unknown reaction pathways.

Asst. Prof. ***Brian Kaspar*** of the Nationwide Children's Hospital and The Ohio State University in Columbus, Ohio and his colleagues transferred the three genes for human follistatin-344, FLRG and GASP-1 with AAV-type-1 vectors locally into single muscles, quadriceps and tibialis anterior, of normal and mdx mice. For comparison, green fluorescent protein was transferred under the same conditions. Injection of 100 billion (10^{11}) AAV1 vectors into 4-week old normal mice resulted, after 725 days (almost 2 years), in an increase of body mass with an observable gross enhancement of muscles not only in those injected, but in others like the triceps also, meaning that this rather local treatment was able to affect other muscles as well. The general muscle force of the entire animal was increased, as measured by the functional grip strength test.

To test an approach more meaningful to a later treatment of Duchenne boys, similar experiments were done with mdx mice using single doses of either 10 or 100 billion virus particles. These dystrophic mice were either three weeks old when injected and then evaluated after five months, or – and this is very important for a later application of this technique to older Duchenne patients – they were injected once when they were 210 days old (seven months), when they already had significant symptoms of their disease, and then followed until they were 560 days old (about 1½ years). 60 days after the injection, they showed increased muscle strength which persisted until the end of the study.

In all these experiments, no obvious safety problems appeared with the virus material or with the therapeutic proteins, follistatin and the other two. The result were robust muscles with increased muscle fiber size, reduced inflammation, and less fibrosis compared to the control-treated mdx mice.

Prof. Kaspar's team finished their publication with the words: "The striking ability of follistatin to provide gross and functional long-term improvement to dystrophic muscles in aged animals warrants its consideration for clinical development to treat musculoskeletal diseases, including older Duchenne patients".

Haidet AM, Rizo L, Handy C, et al. and Mendell JR, Kaspar BK. Long-term enhancement of skeletal muscle mass and strength by single gene administration of myostatin inhibitors. Proc. Natl. Acad. Sciences 2008; 105; 4318-4322.

Inhibition of TGF-beta. The *transforming growth factor beta* (TGFβ) is a protein which inhibits the satellite cells (muscle stem cells) from regenerating muscle tissue. Mdx mice and also Duchenne boys have increased amounts of TGFβ, and this leads to fibrosis (scar tissue), which is caused by the excessive production of connective tissue and its deposition between the muscle fibers in turn replacing degraded and lost fibers. Under normal circumstances, connective tissue holds the muscle fibers together, but increased levels lead to muscle stiffness and contractures. Connective tissue consists mainly of the protein collagen, a rather inelastic molecule that is generated by fibroblasts. Thus inhibiting the activity of TGFβ with drugs may be a possible way to reduce fibrosis.

Prof. ***Andrew Hoey*** of the University of Southern Queensland in Toowoomba, Australia and his co-workers tested *pirfenidone* which is an approved medication for treatment of fibrosis in the lungs. Eight month old *mdx* mice were administered this drug and after seven months of treatment showed reduced levels of TGFβ and restored heart function almost to normal, but fibrosis was not reduced in these old *mdx* mice. The possibility of the drug being more effective in younger mice will be examined in future experiments.

Van Erp C, Irwin NG, Hoey AJ. Long-term administration of pirfenidone improves cardiac function in mdx mice. Muscle Nerve 2006, 34; 727-734.

Losartan and TGFβ. Dr. ***Ronald Cohn*** and his co-workers at the Johns Hopkins University School of Medicine in Baltimore are trying to modify the disease by blocking TGFβ and its signaling pathway that leads to fibrosis. They

started their work with older mdx mice which have a more progressive muscular dystrophy than younger ones. Injection of the snake venom cardiotoxin into non-dystrophic single muscles damages them; they then regenerate within two to three weeks. In mdx muscles, this regeneration is significantly impaired. Treatment of mdx mice with an antibody against TGFβ improves the regeneration time.

As this antibody is not commercially available, the researchers started to test whether *Losartan*, an approved drug against high blood pressure, could have the same effect because it blocks the angiotensin-II receptor, which plays a role in a step further down in the signaling pathway that is initiated by TGFβ. Indeed, it could be shown that treatment of three-month old mdx mice with Losartan for more than one year attenuated many of their dystrophic symptoms like the fibrosis in the muscle and the development of fatigue in muscle function tests. The mice were not cured by this treatment, they were only less sick. Thus, treatment with Losartan, if results with Duchenne boys could be shown to be similar to those with mice, may be a therapeutic strategy similar to other pharmacological treatments that reduce the symptoms without influencing the genetic cause of the disease.

Dr. *Cohn* and his team are now preparing a double-blind clinical trial of Losartan. About 100 Duchenne boys will participate who are between 5 and 15 years old and can still walk. One half of the boys will be treated for one year with Losartan. The other half will receive a placebo for six months and then for the next six month will also be treated with Losartan. All boys in both groups must continue to take steroids if they had taken them regularly before the trial. The primary outcome measure, that is the main test for an effect of the treatment, will be the time they need to walk for 30 feet (about 10 meters). Changes of their quality of life and their respiratory function will be among many other secondary outcome measures.

The researchers hope to start the trial sometime in 2008. The results will then be available about two years later. If the results show a significant therapeutic effect, a recommendation for taking the drug will be issued without delay. However, it is important to emphasize that *until then, parents should not give Losartan to their sick boys*, but should continue their current management including steroid treatment and all additional aspects to keep them in the best possible physical state.

Cohn RD, van Erp C, Habashi JP, et al. Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states. Nature Medicine 2007; 13; 204-210.

Blocking inflammatory agents. The degradation and death of muscle cells in muscular dystrophy causes inflammatory cells to enter the muscle tissue to clean up the cell debris. Steroids are able to suppress inflammation, and this is probably one of the reasons why the drug *prednisone*, its active form *prednisolone*, and the related *deflazacort* can increase muscle mass and strength and reduce the immune response, however often with some uncomfortable side effects. Prof. ***Melissa Spencer*** of the University of California in Los Angeles and her team are performing experiments to find ways to replace the steroids with drugs to counteract inflammation and immune response without their severe side effects.

Studies have shown that some cells of the immune system accelerate the progression of the disease. They produce *cytokines*, molecules that promote inflammation and the development of fibrosis in mdx and Duchenne muscles. In healthy people, this is a normal process of wound healing which stabilizes weak tissue and promotes healing. In Duchenne boys, this healing process does not stop and the muscle undergoes a state of continuous wound healing. Therefore, it is hypothesized that the inhibition of immune cells and active cytokines might slow down the degradation and fibrosis of dystrophic muscles. A number of FDA approved anti-inflammatory drugs already exist which could possibly also act against these immune cells in Duchenne dystrophy. By testing drugs that are already FDA approved, the time to bring these drugs to Duchenne clinical trials will be shortened compared to new compounds.

Three of these drugs currently being used against other diseases are being tested in Dr. Spencer's laboratory on mdx mice: *Galectin-1*, *Remicade*®, and Enbrel®, all against rheumatoid arthritis and *Anti-asialo GM1*, an antibody used in Parkinson's disease.

Osteopontin is a protein which has many functions in bone biology, immune regulation, cell survival, inflammation, and cancer metastasis. Its concentration is increased in the blood and also in the muscles of mdx mice. Dr. Spencer has been examining osteopontin as a potential therapeutic target for Duchenne dystrophy. Mdx mice without osteopontin have better muscle strength, lower CK values, and reduced fibrosis. Work is now ongoing to find a drug which would inhibit osteopontin in Duchenne boys and thus become a candidate for a Duchenne therapy.

Idebenone. The absence of dystrophin does not only weaken the cell membranes but also negatively affects the mitochondria in the muscle cells of Duchenne patients. In these "power stations" of the cells, the universal biological energy carrier, adenosine triphosphate (ATP), is generated by oxidative phosphorylation. The synthetic compound *Idebenone*, developed by the company *Santhera Pharmaceuticals* in Liestal near Basel, under the direction of Dr. ***Thomas Meier***, its Chief Scientific Officer, is chemically derived from the natural coenzyme Q10. Idebenone not only is a powerful antioxidant but, even more importantly, it facilitates the generation of cellular energy. Moreover, Idebenone has been optimized chemically that it can easily enter cells, including muscle cells.

The efficacy of Idebenone has previously been demonstrated for patients with Friedreich's ataxia. In this neuromuscular disease Idebenone was shown to improve neurological function. In addition, Idebenone also could ameliorate hypertrophic cardiomyopathy (enlargement of the heart), which in this neuromuscular disease is a life-threatening complication.

More recently, scientists at the University of Leuven in Belgium, lead by Prof. ***Gunnar Buyse*** in collaboration with Santhera started to determine whether Idebenone would also be beneficial in Duchenne dystrophy. First, they treated dystrophin-deficient mdx mice for 10 months – from shortly after birth until adult age –with Idebenone or placebo in a double-blind study. In this study the cardiac dysfunction of these dystrophic mice was significant-

ly improved. Particularly, the mortality due to experimentally induced cardiac stress decreased from 58% to 19%. In addition, long-term voluntary wheel running performance (an overall muscle function test) improved significantly in mdx mice upon Idebenone treatment. The detailed scientific report of this exciting and innovative study will be published soon.

These results encouraged the researchers to perform a phase-II double-blind, placebo-controlled clinical trial with 21 Duchenne boys at 8 to 16 years of age conducted at the University of Leuven, also under the direction Prof. Gunnar Buyse. Thirteen boys were treated for 12 months with a daily dose of 450 mg Idebenone, applied as 150 mg tablets, while eight patients received placebo. The primary objective of this study was to determine the effect of Idebenone on the heart muscle function measured as the change in peak systolic radial strain of the left ventricular inferolateral heart wall, the region of the heart which in DMD is affected early and most severely. Patients on Idebenone improved markedly on this functional cardiac test compared to placebo. Secondary outcome measures of this study included respiratory function tests. Patients treated with Idebenone improved in peak flow during the 52-week study period, while this respiratory function continued to deteriorate in patients on placebo. These strong indications for improvement in heart and respiratory functions with Idebenone are particularly encouraging, since those problems cause very severe complications in patients with Duchenne dystrophy. Results of this study will be presented for the first time to the medical community at the 2008 annual congress of the American Academy of Neurology.

In summary, this is the first indication of clinical efficacy with Idebenone on cardiac and respiratory functions in Duchenne patients. The results provide the basis for the planning of further clinical development studies with Idebenone. After these positive results, Santhera has decided to continue the development of Idebenone, which already received orphan drug designation, to an effective therapy for Duchenne dystrophy.

Protandim to avoid oxidative stress: The energy carrier of the biological energy every cell needs is adenosine triphosphate, ATP, which is synthesized in the large number of mitochondria inside the cell. They are about as small as a bacterium, and they are using oxygen to synthesize this energy-rich ATP, which also powers muscle contractions. But about 1-2% of the oxygen consumed is converted to the very reactive *superoxide free radical*. The normal cell defends itself against this toxic product with two enzymes: *superoxide dismutase*, SOD, which converts the radical to hydrogen peroxide, and *catalase*, which converts the hydrogen peroxide into water and oxygen.

Muscles cells without dystrophin produce more than the normal amount of superoxide radicals, they experience *oxidative stress*, which then contributes to the degeneration of the muscle fibers because the two enzymes are unable to destroy the excess of the radicals fast enough before they cause chronic inflammation, fibrosis, peroxidation of lipids, a slowing of muscle regeneration, thus the symptoms of Duchenne boys at about three years of age.

For a Duchenne therapy, it would be important to interrupt these processes at this age or earlier. Treatment with the antioxidant vitamins E and C has, unfortunately, no effect. Another possibility is to increase the level of the two enzymes superoxide dismutase and catalase. And in fact, laboratory experiments have shown that the addition of these enzymes to isolated hearts of mdx mice blocks the oxidative stress by destroying the excess of the free radicals and prevents the release of creatine kinase.

For this reason, Prof. ***Joe McCord*** of the University of Colorado developed, together with the company *Life Vantage Corp.* in Denver, the natural formulation *Protandim*® to reduce oxidative stress by upregulation of the two antioxidant enzymes. Protandim contains extracts from five plant species: *Bacopa monnieri*, *Silibum marianum* or milk thistle, *Withania somnifera* also known as ashwagandha, *Curcuma longa* from which the spice turmeric is derived, and *Camellia sinensis* or green tea.

In a clinical trial in 2006, 29 healthy, 20-to-78 year-old persons were treated daily for 120 days. After the end of the trial the two most important test results were that the activity of superoxide dismutase had increased by 30% and of catalase by 54% on the average, and the peroxidation of lipids was significantly inhibited.

Protandim has recently been found to act via the induction of the Antioxidant Response Element (ARE), a genetic mechanism that controls not just the body's production of superoxide dismutase and catalase, but perhaps two dozen other important antioxidant enzymes. Furthermore, the five active phytochemicals in Protandim act together with strong synergy, such that their combined effect is many times greater than the sum of their individual effects.

Nelson SK, Bose SK, et al. and McCord JM. The induction of human superoxide dismutase and catalase: A fundamentally new approach to antioxidant therapy. Free Radical Biol. & Med. 2006; 40; 341-347. Velmurugan K, Alam J, McCord JM, and Pugazhenthi S. Synergistic induction of heme oxygenase-1 by the components of the dietary supplement Protandim. Free Radical Biol. & Med. 2007; 43, Suppl. 1; S97.

Inhibition of NFκB. At Still University in Kirksville, Missouri, Prof. ***George Carlson*** and his colleagues had developed a method to test the calcium influx into isolated severely dystrophic fibers from the expiratory *triangularis sterni* (TS) muscle of mdx mice, to determine whether, as had been assumed, an increased amount of calcium was responsible for the dystrophic symptoms. However, their results showed that this influx was not increased in these stressed fibers compared to the influx into normal muscle fibers under the same conditions. Because other investigators had shown that passive stretch activated the NFκB (pronounced NFkappaB) signaling pathway in muscle, Dr. Carlson's team tried to find out whether this activation was responsible for the damage to their dystrophic TS muscle.

The protein NFκB is present in the cytoplasm of all cells, but is mostly inactivated there by another protein, the inhibitory κB (IκB). Inflammation processes, which combat infections and also cell degeneration, activate the NFκB pathway, and this leads, through a signaling cascade (a chain of reactions) to the upregulation of many genes whose proteins are anti-inflammatory factors. These fac-

tors stop the inflammation when it is not needed anymore. However, if the downregulation of these factors is blocked by genetic mutation or stress situations, the inflammation continues and chronic diseases can develop like arteriosclerosis, lung fibrosis, asthma, rheumatic arthritis, and probably also Duchenne muscular dystrophy.

A number of drugs are available which can prevent the activation of NFκB at the beginning of this signaling cascade. One of these drugs is pyrrolidine dithiocarbamate. Dr. Carlson and his coworkers have tested this drug on their isolated TS muscle fibers from mdx mice and found that their diameter and their function had significantly increased. There are other drugs which inhibit the NFκB pathway that have been already approved for the treatment of diseases other than Duchenne dystrophy. One of these is sulfasalazine. This drug and others are being tested in preclinical trials in order to provide information to conduct clinical trials with them.

Blocking TNFα. The muscle cell membranes of Duchenne boys, which are not stabilized by the dystrophin-protein complex, are easily damaged by the mechanical stress of muscle contraction. Tumor necrosis factor-alpha (TNFα) is a protein which increases the damage to dystrophic muscle by promoting inflammation which leads to necrosis (the destruction) of muscle fibers even in still unaffected muscle tissue. So, blocking TNFα would reduce the degeneration process due to the absence of dystrophin.

Prof. ***Miranda Grounds*** and her co-workers at the University of Western Australia in Perth used an antibody, cV1q, to block TNFα in a long-term study with mdx mice. Older mdx mice have only about 5% of necrotic muscle fibers, and this percentage could not be reduced by treatment with the antibody against TNFα. Thus, the researchers let the mice voluntarily run in exercise wheels, and this doubled the amount of necrotic muscle fibers. Long-term antibody treatment for up to three months, during which time the mice could run as they liked, prevented this additional exercise-induced damage. Therefore, the blockade of TNFα reduces muscle damage as well as the high CK levels normally associated with exercised mdx mice. Also, the cV1q-treated and voluntarily exercised mice ran significantly more than non-treated mdx mice, indicating that they felt well and that their muscles had improved function. Clinical trials with this antibody or with other known drugs that block TNFα should be considered.

Upregulation of IGF-I. The insulin-like growth factor (IGF-I) is a protein with about 70 amino acids in one chain with three stabilizing bridges, thus with a similar shape as insulin. It exists in multiple forms with slightly different structures. One of these so-called isoforms, IGF-1A, is very beneficial for muscle, because it helps to promote growth and strength and is of interest for a possible therapeutic use in Duchenne children.

The research team of Prof. ***Elisabeth Barton*** of the University of Pennsylvania in Philadelphia works with mdx mice which were genetically engineered, so that they produce high levels of IGF-I in their muscles throughout their lifetime. These *mdx-IGF-plus mice* show an increased muscle growth with quite healthy-looking muscles and much less fibrosis than the "normal" mdx mice.

But because this growth factor interferes with many processes in other than muscle cells, potentially serious side effects cannot be excluded if higher dosages are used to optimize the effect on muscles. Therefore, IGF-I was complexed with one of its binding proteins (IGFBP3) to produce IPLEX™, an already approved drug that stabilizes IGF-1 in the blood, and releases it only where and when it is needed. A first clinical trial with IPLEX was performed at University of Rochester with 15 adult myotonic dystrophy patients. This strategy could be very effective in getting IGF-I to the muscle without causing side effects in other tissues.

Another way to create higher levels of IGF-I in muscle tissue would be to transfer its gene into muscles with AAV vectors, which would instruct them to make more IGF-I. Work with this technique in Dr. *Barton*'s laboratory succeeded in increasing the level of the most active isoform IGF-IA 30 to 40 fold after intramuscular injection of the vectors. The newly synthesized IGF-I stayed in the muscle tissue, promoted hypertrophy (enlargement of muscle fibers), but avoided side effects caused by the activation of non-muscular tissues. Such a viral gene therapy will take several years until it could be tried in Duchenne boys.

Beta-agonists. Beta-agonists are hormone-like substances that bind to specific receptor proteins on the outside of cell membranes and then start a chain of chemical reactions, a *beta-adrenergic signaling pathway* or *cascade*, to deliver a signal to biological targets inside the cell which are important for controlling protein synthesis and protein degradation. Some beta-agonists are approved drugs like *bronchodilators* to relax the airway muscles of asthma patients, or used as *anabolic agents* to improve the size and strength of skeletal muscles, sometimes used illegally by athletes ("doping").

In the laboratory of Prof. ***Gordon Lynch*** of the University of Melbourne, the beta-agonist *formoterol* was tested with excellent results for its ability to reverse muscle wasting in old rats, a finding with clinical potential to treat older people. These positive results suggested that such "anti-aging drugs" could also be used for a potential therapy for Duchenne muscular dystrophy.

In fact, a small clinical trial with Duchenne and Becker dystrophy patients has already been performed. The participants were treated for 28 weeks with *albuterol* (8 mg/day) another beta-agonist which is approved for asthma. This low dose was chosen after another one-year trial with adult FSH dystrophy patients (another muscle disease) had shown that at doses of 16 and 32 mg/day, albuterol led to some unacceptable heart problems such as palpitations. The reduced dose in the trial with DMD patients did not cause any side effects but produced only a modest increase of muscle strength which was insufficient for an effective therapy against muscle wasting and weakness.

To prepare another clinical trial with a more powerful beta-agonist, Prof. Lynch and his colleagues treated *mdx* mice with very low (clinically-relevant) doses of *formoterol* (25 micrograms/kg). This low-dose increased the size and strength of fast-twitch and slow-twitch muscles of the mdx mouse and importantly did not make the muscles more easily fatigued. The effects on the size of the heart were also reduced with low-dose treatment.

As Duchenne patients do not need an enlargement of their hearts, the effect of these drugs on the heart muscles must be avoided while maintaining the positive effects on the skeletal muscles. Separating these two effects is still an important scientific challenge and this is a major focus of Prof. Lynch's research with these compounds. Another side effect, the downregulation of the receptors for the beta agonists in the muscle cell membranes, which reduces their effect on skeletal muscles, must also be avoided before clinical trials for a long-term treatment of Duchenne boys can be started.

Lynch GS, Ryall JG. Role of β-adrenergic signaling in skeletal muscle structure and function: implications for muscle wasting and disease. Physiological Reviews 2008; 88: 729-767.

Harcourt LJ, Schertzer JD, Ryall JG, Lynch GS. Low dose formoterol administration improves muscle function in dystrophic *mdx* mice without increasing fatigue. Neuromuscular Disorders 2007; 17; 47-55.

BBIC inhibits proteases. The degradation of muscle proteins in Duchenne dystrophy is caused by several different proteases (protein-destroying enzymes) among them the enzyme *calpain* and a large protein complex, called the *proteasome*. In Duchenne dystrophy, muscle cell membranes become leaky, so that calcium ions (charged atoms) can enter the cells and activate calpain and also the proteasome. Researchers are trying to inhibit the activation of calpain and other proteases and thus delay muscle cell degradation.

Prof. ***Lee Sweeney*** and his co-workers at the University of Pennsylvania in Philadelphia are experimenting with one of the protease inhibitors, the *Bowman-Birk inhibitor concentrate*, (BBIC), a natural protein composed of 71 amino acids, which can be isolated from soybeans. It is a water-soluble substance that can be orally taken. As it is too big to enter the muscle cells, it blocks several proteases like the digestive enzymes trypsin and chymotrypsin outside the cells and interrupts signaling pathways that can produce inflammation processes in Duchenne dystrophy. Long-term treatment with BBIC increases the muscle mass and strength in mdx mice. CK activities are reduced considerably and fibrosis also. From other applications in cancer patients, it is known that BBIC is a very safe drug.

A clinical phase-I trial is now being prepared together with Dr. *Kenneth Fishbeck* at the National Institutes of Health (NIH) in Bethesda near Washington. If the trial shows that similar results as those found with mdx mice can be obtained in Duchenne boys, this rather benign drug can possibly slow down their muscle degradation. Soybeans contain other proteases also, so BBIC must be isolated and purified from them. Eating the beans directly has no effect.

GAMT and AGAT. Although the mdx mouse has no dystrophin in its muscles, it does not show the severe clinical symptoms of human Duchenne muscular dystrophy. Prof. ***Brian Tseng***, formerly at the University of Colorado in Denver and now at the Harvard Massachusetts General Hospital in Boston and his team continue to investigate ways to slow down muscular dystrophy.

The scientists used screening techniques to find "modifier" genes that are upregulated in mdx mice but downregulated in Duchenne boys. They identified two such *genes* for the enzymes *arginine:glycine amidotransferase*, AGAT, and *guanidinoacetate methyltransferase*, GAMT. Both are important for the synthesis of creatine, which is required for biological energy in skeletal muscle. Unlike Duchenne boys, the mdx mouse can upregulate both enzymes so iot can make its own creatine in its muscle cells. It is known that Duchenne boys have only 20% of the normal amount of creatine found in healthy muscle. In contrast, the mdx mouse has 80-90% of creatine in their muscles compared to healthy control mice. An mdx mouse was created whose GAMT gene was genetically inactivated. This mouse cannot walk well, dies early, and its muscles look more severely affected, similar to those of Duchenne boys.

Now, the researchers in Dr. Tseng's laboratory are working to create an mdx mouse without the other enzyme, AGAT, which may be severely handicapped, too. And they are investigating how the absence of dystrophin protein seems to interfere with the transport of creatine from the bloodstream into the muscle cells, an effect which may speed up muscular dystrophy. This creatine transporter problem cannot be as effectively treated by dietary creatine alone. Dr. Tseng's team is also studying the two genes GAMT and AGAT in boys and men with an atypical muscular dystrophy, who have no dystrophin but Becker-like symptoms and near normal muscle strength.

With high throughput screening methods, it may be possible to identify compounds, approved drugs, or "nutriceuticals" (effective dietary substances), which could upregulate the two "modifier" genes GAMT and AGAT in Duchenne boys. This could make their severe symptoms more like those of the hardly handicapped mdx mouse and buy time while efforts for a "real cure" continue.

McClure WC, Rabon R, Ogawa H, Tseng BS. Upregulation of the creatine synthetic pathway in skeletal muscles of mature mdx mice. Neuromuscular Disorders 2007; 17; 639-650.

L-arginine and nNOS. Another consequence of the missing dystrophin is the reduced amount of one particular component of the dystrophin-glycoprotein complex, the enzyme *neuronal nitric oxide synthase* (nNOS). This enzyme produces nitric oxide (NO) from the amino acid L-arginine. Although NO is a gas, it acts like a hormone and regulates, among other effects, the dilation of blood vessels which is important for the normal supply of blood and therefore of energy to the muscles. When nNOS is missing, cardiac fibrosis develops and this may contribute to increased fibrosis in hearts of *mdx* mice and also of Duchenne patients.

Prof. ***Andrew Hoey*** and his colleagues at the University of Southern Queensland in Australia administered L-arginine daily for 6 months commencing in 6-month old mdx mice. This reduced the fibrosis in their hearts, increased the coronary blood flow and improved their heart function. In ongoing experiments, the mechanism of this effect of L-arginine is being investigated before much further work can be done to see whether L-arginine can become a drug for Duchenne boys.

Some final thoughts about where we are and what should be done.

As I said at the beginning, I wrote this research report and my earlier ones especially for you, the boys and young men with Duchenne muscular dystrophy and your families, so that you better understand what is being done to stop this disease, to *end it* once and for all. I have selected 33 research projects for my summaries, because they are, in my opinion, the most important ones with which dedicated scientists in many countries are trying to find effective and safe ways to stop or to at least slow down the steady disappearance of your muscles.

Good and bad mutations. Duchenne muscular dystrophy is not new, like AIDS, because it has been seen in mice, rats, cats, and dogs, and thus probably exists in all animals with muscles. So it started long before we became different from our animal ancestors. It is an accident of evolution. Without mutations, the random changes of the genetic information, we would not be here and the rest of life not either. Some of the mutations are "good", because they improve life, but most are "bad" and dangerous, because they cause disease before their birth or afterwards.

The mutations that cause Duchenne muscular dystrophy do not punish you or anybody else for something, they just happen. This is not the place to discuss religious questions which easily come to mind. Just allow me to add one thought: Nature seems to act blindly without regard to whom she hurts, on the other hand, her good mutations gave us the brains to find a way for repairing this terrible side effect of creation.

Scientific research. If you have read the entire report, you will realize that the many scientists and their teams mentioned are doing everything they can as fast as possible for finding a therapy. In 1986/87, when the dystrophin gene and its protein were found, we all thought that the way was finally open for a cure that could very soon correct the molecular-genetic cause of the disease. But now, more than 20 years later, we are still waiting for that cure or at least for a therapy that would slow down the destruction of the muscles. But not only the fight against *this* disease proved to be much more difficult than first imagined, progress for other genetic diseases like cystic fibrosis or the many forms of cancer, is also very slow. In fact, "our disease", Duchenne muscular dystrophy, could become the first rare hereditary disease which will have a genetic therapy in the not so distant future.

One step after the other. The research approaches explained in this report are all based on reliable scientific results, so most will sometime lead to techniques that will materialize in drugs that can be bought. But it will take time, because their development will have to proceed step by step, and every one of them must be carefully planned, tried in animals and then finished with clinical trials. Shortcuts, even if they look possible in theory, are not allowed. After all, our children will have to take most of their Duchenne drugs for the rest of their hopefully long life. So they cannot have side effects that accumulate and get dangerous with time. *They have to be absolutely safe.* The scientists know this, and they also know that the regulatory agencies like FDA and EMEA, although they often seem to make their work more difficult and to slow it down, are there to protect you. The step-after-step process is slow, but mistakes with accidents that hurt you or others with other diseases would only slow down the development of our and their drugs.

Exon skipping. One of the genetic techniques discussed in this report is especially promising: *exon skipping.* In a clinical trial in the Netherlands, one kind of the potential antisense drugs, AONs, has been shown to work in one muscle of four Duchenne boys. A similar trial with another kind of these drugs is now being performed in the UK. There is justified hope that its results will be equally positive. Systemic trials are now planned or already underway with the injection of the AONs into the blood circulation so that they can reach all muscles. That worked in animals, and we are all hopeful the results will be the same in children.

The participating boys in these systemic trials, who need skipping of exon 51, will have that exon removed in a large part of the damaged genetic messengers, mRNA, of their dystrophin. Their muscles might already work better at the end of the trial. Thus, we hope this approach will become *an effective therapy*, even if it is not a complete cure. It will not take another 20 years until it his here, it will be much earlier.

An exon skipping treatment will have to be repeated after some time, some weeks or months. But this has the advantage that it can be stopped and replaced by some other more effective or more permanent method. Exon skipping will be a great help for many Duchenne boys, but it will only slow down the progression of the disease, thus will be an effective therapy but not a complete cure.

Pharmacological approaches. So, for many of you who are still young and thus have much of your muscle tissue left, exon skipping will not come too late. But lost muscles cannot be repaired by exon skipping. Therefore, the older ones among you will possibly benefit more from the pharmacological research approaches than from the genetic ones. Some of these drugs can be misused for doping of athletes, but the possible benefits for you with muscular dystrophy means they should be allowed for this application.

The list of these potential drugs is long, about 30 are discussed in this report and more will appear in later updates. Some of them are in clinical trials with good results like Idebenone. They might be fully developed faster than the genetic methods. And either alone or together with others, in *cocktails*, they may maintain and strengthen the muscles, like the steroids but with fewer side effects, and thus *buy time* while you are waiting for a more fundamental genetic therapy.

Clinical trials are indispensable steps to the full development of a therapy. But they are just human experiments that also can go wrong. And some already had negative results, like Myodur that did not stop proteases and Myo 029 that was supposed to inhibit myostatin but did not lead to clinical benefit. And the first of a series of clinical trials, those of phase-I, are not expected to provide a clinical benefit. Most try to treat only one unimportant muscle, and this cannot improve the disease of all muscles. Are the po-

tential new drugs safe? That is the main question these trials are designed to answer. Participation is important, but it is not worth to come with great expense from far away to the trial centers.

Mutation diagnostics. Some potential therapies are mutation specific that means the exact mutation must be known for the patient to benefit from these treatments. Examples are exon skipping and PTC124. Others, like the replacement of the dystrophin gene with virus vectors or the upregulation of utrophin are independent of the mutation. The MLPA method is now a widely used technique for detecting deletions and duplications in boys and also in female carriers. However, it is expected that that new microarray techniques will soon replace all other gene testing methods.

Carrier diagnosis for women related to the mother of a Duchenne patient is important for their genetic counseling which can avoid the birth of additional Duchenne boys in the extended family of a patient. But if a woman at risk can be assured that she is *not* a carrier, this can encourage her to have healthy children without fear of a recurrence.

Newborn screening for high CK activity in dry blood-spots, as it is offered in Germany (Freiburg), Wales (Cardiff), and Belgium (Antwerp), finds Duchenne boys early and may, through genetic counseling, avoid the birth of secondary cases in the same family. Two pilot CK-screening programs are now underway in the US in Columbus/ Ohio and Atlanta/Georgia.

Registration. All boys and young men with Duchenne should have their personal medical data registered in the Duchenne data banks of their own country which should be part of the international registry networks as offered by TREAT-NMD (www.treat-nmd.eu/registry) and DuchenneConnect (www.duchenneconnect.org). This would allow finding participants for clinical trials of therapies for more unusual mutations, and it would also assure that the patients and their families have access to the most up-to-date information about research results and medical management.

Get together. You, the families with Duchenne boys and the young Duchenne men themselves should become part of the worldwide Duchenne community and work actively there. You should get together with other families and patients in the muscular dystrophy associations of your own country and also on the international level. The EAMDA, European Alliance of Muscular Dystrophy Associations, is one example. Together you can do many things to speed up the development of therapies. Here are some suggestions:

Faster approval. The FDA and other regulatory agencies need many months to approve clinical trials of new techniques. They have finally approved the local exon-51-skipping trials, but it took them about a year to approve the British trial. That was the reason why the Dutch could finish their trial before the British were even allowed to start. Now it looks as if these agencies would insist on approving every one of the AONs for the many different deletions and duplications. The scientists will try to convince them that only the sequences of the AONs will be different, but everything else including the chemistries will be as the first ones for skipping exon 51. You, the families, should Work together with PPMD and other organizations and start open discussions with the regulatory agencies. Such discussions have already begun with the FDA and are progressing quite well. If successful, the many necessary skipping drugs for the exons other than 51 would be available within months and not years after the first one for skipping exon-51 is ready for you.

Educate your doctors. Educational materials are available on DuchenneConnect, TREAT-NMD, PPMD, ActionDuchenne and UPPMD. It is important to utilize these materials, which are available on-line and are updated frequently, when speaking with your physicians. Many of your local or primary care doctors will have little, if any, familiarity with Duchenne. It is important to educate them to help them to care for your child in an informed and understanding way.

Beware of miracle drugs and treatments. It should have become clear after you read this report and perhaps listened to the presentations and discussions of "our" scientists at the many Duchenne meetings, therapeutic drugs and treatments that are safe and effective for a long time can only be produced after careful development with strictly scientific methods. If you see miracle drugs on the internet or get offers of miracle treatments, and you consider getting or applying them for your child, please ask the miracle providers how many Duchenne boys they have already cured, how these boys were diagnosed with what results, how much new dystrophin have they found in the muscles, and how much the muscle function has improved. If that what is offered really had any value, ask yourself why not thousands of families are going there with their sick children. Be careful, otherwise you will lose lots of money and possibly hurt your boy severely.

Promote awareness. You should work actively with your muscular dystrophy associations in your countries and they should get together with international organizations like TREAT-NMD in Europe and PPMD in the United States and in several other countries. All of us together will be able to bring your day-to-day problems and your hopes for a treatment to the attention of your governments and the general public, to the other people who have no idea what Duchenne means. This could be done by political lobbying and with help of the media, newspapers, radio and television. This would open the eyes of the granting agencies and charities to where their research money is needed, and it would also bring tax-deductible donations from private sources.

Donations for research, even small ones, are important. They might only contribute a fraction of the real costs of drug development which go into the millions of dollars, pounds or euros. These pennies and cents are a reminder of the not-so-rich people to the real-rich ones that they care for you too and make it happen that you, like everybody else, can have a long and happy life.

Links to other important articles in earlier reports.

In my earlier reports on the three Parent-Project meetings of 2006 and 2008, many more subjects were described which are not mentioned in this report on the research approaches. They are listed here with an indication where they can be found. You can see these earlier reports on my internet pages www.duchenne-information.eu in English after clicking on "Reports on the research for a therapy of Duchenne muscular dystrophy". The short names of the reports "2006 Cincinnati English" (C06), "2006 London English" (L06), and "2007 Philadelphia English" (P07) are further abbreviated in the following list as in the parentheses followed by the page number (e.g. P07-20) for the article referred to.

Nick Catlin: Standing on the shoulders of giants (L06-1). ***Louis Kunkel:*** Twenty-year anniversary of finding the dystrophin gene and its protein (L06-2).

Robert Weis: Why do we need to know the exact mutation (P07-19)? ***Stephen Abbs:*** Why should one test for the mutations in the dystrophin gene (L06-17)? ***Kevin Flanigan:*** If there is still no cure, why do we need to know the exact mutation (C06-13)?

Jennifer Morgan: Viral vectors and muscle stem cells (L06-7). ***Terence Partridge:*** The promise of stem cells (C06-7).

Kate Bushby: Why do we need clinical trials (P07-3, L06-3)? ***Diana Escolar:*** International clinical trials (P07-16). ***Kate Bushby:*** North Star Project, international clinical trial with steroids (L06-12).

Tan Nguyen: How does the FDA approve a Duchenne drug (P07-17)? ***Robin Sharp:*** Duchenne registry (L06-18).

Serge Braun, Kate Bushby: TREAT-NMD, a network of excellence of the European Union (P07-18). ***Madhuri Hedge:*** CETT, collaboration, education and test translation program (P07-20). ***Kyle Brown:*** DuchenneConnect will encourage collaboration and genetic testing for Duchenne muscular dystrophy (P07-21). ***Patricia Furlong:*** What does "Connect…" mean (P07-22)? ***Francesco Muntoni:*** Do not miss the wood for the trees (L06-19).

Steve Wilton: How exon skipping works (L06-4). Steve Wilton: An interview on exon skipping (C06-14). ***Gertjan van Ommen, Francesco Muntoni:*** When will there be an exon-skipping drug for Duchenne boys (P07-8)?

I have written this report on behalf of TREAT-NMD and PPMD, whom I thank for financial support and whom you can contact directly:

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This report will be updated repeatedly, the next time after the annual meeting of PPMD in Philadelphia, 17 to 20 July 2008. You can see this report and its translations into German and Spanish on the internet at www.duchenne-information.eu as well as the English, German, and Spanish versions of my reports about the two PPMD meetings in Cincinnati 2006 and Philadelphia 2007 and the ActionDuchenne meeting in London 2006. If you wish to receive all my future reports as soon as they are ready, please send me your e-mail address for inclusion in my English, German, or Spanish mailing lists which already contain more than 1,000 addresses.

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Molecular details of skipping exon 51.

In the clinical trial in the Netherlands, skipping of exon 51 has been achieved. Here, the molecular details of this skipping are explained whose aim was to restore the reading frame which was shifted in the mRNA by the deletion of exon 50 in the dystrophin gene.

Part of the base sequences of exons 50 and 51 of the mRNA of the *normal dystrophin gene* are shown as well as the end of exon 49 and the beginning of exon 52. In exon 50, 29 triplets are not shown and 52 in exon 51. Below each triplet, the abbreviation of the name of the amino acid in the dystrophin protein is shown that is coded by the triplet. The triplets follow each other without spaces, the hyphens indicate only the reading frame and the vertical lines the borders of the exons. The three bases of the *hidden* stop signal UGA are shown in red. Exon 50 ends after the first base of the last triplet, which then is completed to UCU with the first and second bases of exon 51, shown in blue.

```
        End Exon 49 | Start Exon 50        End Exon 50 | Start Exon 51
---CAG-CCA-GUG-AAG | AGG-AAG-UUA-GAA---AUU-GGA-GCC-U  | CU-CCU-ACU-CAG-ACU-
   gln pro val lys | arg lys leu glu   ile gly ala ser|    pro thr gln thr

              hidden stop codon
---GUU-ACU-CUG-GUG-ACA-CAA---AAA-CUA-GAA-AUG-CCA-UCU-UCC-UUG-AUG-UUG-GAG---
   val thr leu val thr gln   lys leu glu met pro ser ser leu met leu glu

                          End Exon 51 | Start Exon 52
              ---AUG-AUC-AUC-AAG-CAG-AAG | GCA-ACA-AUG-CAG-GAU-UUG---
                 met ile ile lys gln lys | ala thr met gln asp leu
```

When exon 50 is deleted in the gene and also in the mRNA, exon 49 is followed directly by exon 51. This causes the shift of the reading frame in exon 51 by one nucleotide to the right, with the consequence that 8 incorrect amino acids are incorporated into the dystrophin until finally a premature stop signal UGA is reached. The shifted base sequences and the wrong amino acids are shown in red. The synthesis of dystrophin is interrupted prematurely, it remains incomplete, is destroyed, and *Duchenne muscular dystrophy* develops.

```
                        End Exon 49 | Start Exon 51
                   ---CAG-CCA-GUG-AAG | CUC-CUA-CUC-AGA-CUG-UUA-
                      gln pro val lys | leu leu leu arg leu leu

   active stop codon                    antisense oligoribonucleotide
                                        UC-UUU-ACG-GUA-GAA-GGA-ACU
-CUC-UGG-UGA-CAC AAG---AAC-UAG-AAA-UGC-CAU-CUU-CCU-UGA-UGU-UGG--
 leu trp STOP!

                        End Exon 51 | Start Exon 52
              ---AU-GAU-CAU-CAA-GCA-GAA-G | GC-AAC-AAU-GCA-GGA-UUU---
```

The exon-skipping antisense oligoribonucleotide, AON PRO051, as used by the Dutch researchers, is shown in blue attached by Watson-Crick base pairing to 20 bases in exon 51. It induces skipping of exon 51 in the mRNA of the *mutated* gene which, in this example, does not contain the sequence of exon 50.

If, in addition to the deleted exon 50, exon 51 is removed by skipping, then exon 52 is directly connected to exon 49. The reading frame is not disturbed any more because exon 49 ends and exon 52 begins with a complete codon of three bases.

```
                        End Exon 49 | Start Exon 52
                   ---CAG-CCA-GUG-AAG | GCA-ACA-AUG-CAG-GAU-UUG---
                      gln pro val lys | ala thr met gln asp leu
```

No premature stop signal appears in exon 52 or later, but 77 amino acids are missing in the protein, those whose genetic information was carried by the base sequence of eons 50 and 51. They are missing in the central part of the shortened dystrophin, which, however, will probably still be partly functional and thus give rise to the mild Becker dystrophy instead of the severe Duchenne dystrophy.