

## Muscular Dystrophy Annual Conference, 13 - 16 July 2006

### Approaching Therapies for Boys with Duchenne Muscular Dystrophy.

**Face it! Live it! Change it!** This was the title of the Parent Project Muscular Dystrophy (PPMD) Annual Conference which took place from July 13 to 16, 2006 in Cincinnati/ Ohio. Thirty-six scientists and clinical experts for muscular diseases presented, first, reviews about the entire knowledge of Duchenne muscular dystrophy and about the different approaches for a therapy (*Face it!*), then recommendations for the medical management of the our sick boys (*Live it!*), and, finally, their latest research results that come closer and closer to an effective treatment (*Change it!*). I, *Günter Scheuerbrandt*, a biochemist from Germany, was asked by *Patricia Furlong*, the founder and president of PPMD, to write this report for you, the boys and their families, who wish to know about each successful step on the way to an effective treatment.

The report contains the summaries of only the scientific presentations because I am not a clinical expert. It is not a scientific publication, it is written in a language you will hopefully understand. The most important scientific expressions are explained at the beginning and the others later, when they are first used.

All scientists whose presentations are part of this report, have had the opportunity to see the draft of my text and to correct it if necessary, and all of them have done so. Thus there should be very few mistakes left.

At the beginning of the summaries, I am using the names of the scientists without their titles, most are professors and all have either a PhD or MD title or both. And almost all are heads of laboratories, that means 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.

This terrible disease, Duchenne muscular dystrophy, is slowly loosening its grip, this was obvious after we heard the presentations on so many new research results. It is being conquered step by step by dedicated people working for us in many countries. The following text will show you why.

### Introduction

The report begins with my paragraphs on some basic facts to understand how genes make proteins, why dystrophin is so important, which research approaches a being actively followed, and how exon skipping works, presently - in my opinion - the most advanced of these approaches. At the meeting, *Lee Sweeney* of the University of Pennsylvania in Philadelphia, *Dominic Wells* of the Imperial College in London, and *Steve Wilton* of the University of Western Australia in Perth gave quite detailed introductions of these four subjects. I included many of their explanations, but not all in these first paragraphs without keeping the contributions of each of them apart. After this introductory section, each scientist's presentation is summarized separately, in some cases with background explanations.

**How do the 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 consists of 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 types of base combinations, the **base pairs** A-T and G-C. Therefore, the **sequence of the bases** on one DNA strand is *complementary* to the sequence on the other. This sequence of the bases, of the "genetic letters", is the genetic information for the development and maintenance of a living organism that 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 of "inactive" ones, the often much longer **introns**, which, however, may be important 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 outside the nucleus. The ribonucleic acids, **RNAs**, 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 and 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 acids**, 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 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 skeletal muscles. The gene has seven or possibly eight different **promoters**, base sequences to which regulating proteins bind and thus activate the gene by allowing the transcription of its information to finally produce its protein. Because of the many promoters and by alternative splicing, many additional *different forms of dystrophin* exist, all of them are shorter than the normal one in the muscles. These are located in different organs, one of them in the brain. It is only 32% as long as the normal one, and it also can be affected by mutations. This may be the reason for mental problems of some Duchenne boys.

**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 about 25,000 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 is much shorter than its gene, it is 125 nm (= 0.000125 mm) long, 80,000 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 the disease, to let the muscles function again, a large percentage 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 or like a shock absorber. Thus dystrophin transmits the mechanical energy produced by the actin-myosin "contraction apparatus" to the muscle cell membranes and the structures outside them, the connective tissue and the tendons, in a well-balanced way that does not overstress 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. The consequences are a chain of events like inflammation and activation of fibroblasts which lead to **fibrosis**, scar tissue, which slows down muscle regeneration and causes the typical symptoms of older Duchenne patients.

Boys with the slower progressing Becker dystrophy mostly 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, as *Andrew Hoey* discussed, 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 gastro-intestinal tract is affected when the motility of the intestines is reduced. So the change in just one gene can affect the whole body.

**The mutations of the dystrophin gene:** There are three common types of mutation that affect the function of the dystrophin gene: **Deletions**, if one or more entire exons of the gene are missing, **duplications**, if parts of the gene are repeated, and **point mutations**, if single base pairs are exchanged, eliminated or added. Others are inversions and mutations in the 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 **reading frame** is not disturbed, when the mutation deleted or added 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 dystrophy**.

If, however, the mutation shifted the reading frame by one or two base pairs, the reading frame becomes **out-of-frame**. Then, a number 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 fulfil its normal function, it disappears and **Duchenne muscular dystrophy** develops. At the end of this report, the appearance of a premature stop codon is shown in an example of exon skipping.

## The different strategies for a therapy of Duchenne muscular dystrophy.

Research tries to develop a therapy for Duchenne muscular dystrophy with two genetic approaches or with many different pharmacological interventions.

The first genetic approach is the attempt to introduce new dystrophin genes into the nuclei of the muscle cells which then could again direct the production of dystrophin. Many experiments in mice have shown that this can be achieved by using a modified, a "tamed", virus, the **adeno-associated virus**, AAV, as a transporter or **vector** to transfer the active parts, the combined exons - the cDNA - of the dystrophin gene into the muscle cells. But the AAV vector is not large enough to carry the complete cDNA with all 79 exons. Only cDNAs one third as long as normal would fit in them. This means that the new dystrophin will also have only one third of its normal size. If this shortened dystrophin has one of the structures causing the benign Becker dystrophy, the effect of such a treatment would not be a complete cure but a slowing down of the fast Duchenne type dystrophy to the more benign Becker form with a practical normal life expectancy. As the newly introduced genetic material does not enter the chromosomes of the cell, the mutated dystrophin gene is not changed, it remains as it was on the short arm of the X chromosome.

The second genetic approach, **exon skipping**, also does

not touch the damaged gene. It only interferes with the processing of the genetic information on its way from the gene to the protein. The splicing of the exons of the pre-mRNA to the mRNA is specifically altered so that the disrupted, *out-of-frame* message is made readable again, made *in-frame*. The result is the same as with the gene transfer technique: Duchenne dystrophy is slowed down to Becker dystrophy. A completely new type of medication, "genetic drugs", specially designed for each patient, can do this information altering: **antisense oligoribonucleotides**.

As both genetic approaches are new, research must proceed very cautiously. Although it is tempting to push new therapies to the clinic rapidly, it is important not to make the mistake of compromising safety as this would set back the entire genetic-therapy field. Therefore, the approval procedures are very strict and take much time.

The third therapeutic approach tries to combat the non-genetic consequences of the absence of dystrophin like muscle destruction by protein destroying enzymes, leaking membranes, fibrosis, and inflammation. There are a number of drugs, some of them already on the market against other diseases, that are expected to have beneficial effects on Duchenne dystrophy. In the section on **pharmacological approaches** the most recent research results are summarized, which were discussed at the meeting,

## Exon Skipping

The **exon skipping** technique tries to change a Duchenne mutation into a Becker mutation. If a mutation disturbs the reading frame and thus causes Duchenne dystrophy, the reading frame can be **restored** by artificially removing from the messenger RNA one or more exons directly in front or after the deletion, the duplication, or the exon which contains a point mutation.

Exons can be eliminated from the mRNA with **anti-sense oligoribonucleotides**, AONs. They are short RNA single-stranded structures consisting of 20 to 30 nucleotides whose sequences are constructed in such a way that they attach themselves only at the complementary sequence inside the exon to be removed or at its borders *and nowhere else*. *Antisense* means that their base sequence is in reverse order to the target sequence in the pre-mRNA. These AONs thus interfere with the splicing machinery so that the **targeted exons** are no longer included in the mRNA, they are *skipped*.

*The gene itself with its mutation is not altered by exon skipping*, but its mRNA no longer contains the information of the skipped exon or exons. As this 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 central rod domains, the shorter protein can often still perform its stabilizing role of the muscle cell membrane. The result would be the

change of the severe Duchenne symptoms into the much milder symptoms of Becker muscular dystrophy.

**Oligonucleotides** are short pieces of the two kinds of nucleic acids, DNA and RNA - oligo means few. The two strands of **DNA**, deoxyribonucleic acid, consist each of a chain of alternating phosphate and deoxyribose units, their backbone. **Deoxyribose** is a sugar molecule with five carbon atoms, and the second carbon atom has its usual oxygen atom missing. Each sugar unit carries one of the four bases on its first carbon atom. **RNA**, ribonucleic acid, has normal **ribose** units in its backbone with an oxygen on its second carbon atom. **Nucleotides** are the building blocks of both kinds of nucleic acids. Each nucleotide consists of one ribose, one base and one phosphate. So there are four different **ribonucleotides** and four different **deoxyribonucleotides**.

The two kinds of AONs mostly used for exon skipping are protected oligoribonucleotides so that they are not or only slowly destroyed in the muscle cells by nucleases, enzymes, that destroy nucleic acids.

The Dutch scientists are using **2'-O-methyl-phosphothioates**, also called *methyl thioates* or *2O-methyls*. They have a methyl group, a carbon with three hydrogen atoms, on the oxygen of the second carbon of the ribose units, and a sulfur atom instead of one of the oxygen atoms of the phosphate groups. The **morpholinols**, the British and Aus-

tralian researchers are using, have one of the phosphate oxygens replaced by a dimethyl amide group, a nitrogen carrying two methyl groups, and the entire ribose units are replaced by morpholino rings, six-membered rings, each consisting of four carbon atoms, one oxygen and one nitrogen atom with hydrogen atoms attached to the carbons.

Many more details about exon skipping are discussed in the interview with Dr. *Wilton* at the end of this report. And on the last page, the skipping of exon 46 to restore the reading frame after the deletion of exon 45 is shown as an example of this technique.

**Exon skipping: The first clinical trials with Duchenne boys in the Netherlands.** **Prosensa B.V.** is a biotechnology company in Leiden in the Netherlands currently developing a therapy for Duchenne muscular dystrophy using the exon skipping technique in cooperation with Drs. *Gertjan van Ommen* and *Judith van Deutekom* of Leiden University Medical Center. **Gerard Platenburg**, Prosensa's president, announced that on 8 May 2006, the two Dutch regulatory committees - CCMO and IRB - gave permission to "an exploratory study on the efficacy, safety, and tolerability of a single intramuscular dose of antisense oligoribonucleotide, AON, to restore production of dystrophin". This opened the way for *the first in-human exon skipping trial* which is now beginning.

For this trial, six Duchenne boys, 8 to 16 years old, all from the Netherlands, have been selected. They have a dystrophin-gene mutation that causes a frame-shift which could be restored by skipping of exon 51. After intensive clinical tests, including skin biopsies, the potential drug, a so-called 2O-methyl AON directed against exon 51 will be applied in one injection into one single muscle, the tibialis anterior muscle of the shin. The patients will be injected sequentially, i.e. only after no side effects appeared in one boy will the next boy be treated. Four weeks after the injection, a muscle biopsy will be taken and the material analyzed for the shortened dystrophin protein. The main objective of this first trial is to prove that exon skipping is safe and works in Duchenne patients as expected after the many successful pre-clinical experiments with muscle cultures and animals. Even if new dystrophin is found in the one treated muscle, the boys will not have any therapeutic benefit from this local treatment.

The Dutch researchers have selected the 2'-O-methyl-phosphothioate AONs, also called 2O-methyls, because they have extensive experience with this type of AONs, not only with injections directly into the muscle tissue, but also with the systemic application into living animals. For instance, after repeated injections of the 2O-methyl against mouse exon 23 into the tail vein of mdx mice, substantial therapeutic amounts of the skipped dystrophin appeared in all skeletal and heart muscles.

The fact that the 2O-methyls enter also the cardiac muscles is important, because it has not been possible yet to have the other kind of extensively tested AONs, the morpholinos, get into the heart.

It has also been shown that the 2O-methyls are taken up more readily by dystrophic than by normal muscle cells, presumably because there are "holes" in the dystrophic membranes. In the mdx mice, the effect of the systemic skipping with 2O-methyls lasts several months, which is an indication that a future exon skipping therapy would

possibly have to be repeated every month. However, the exact dose and dosing frequency remains to be determined.

Because of the very promising pre-clinical systemic results, the Dutch researchers are already preparing the next clinical trial with Duchenne boys scheduled for 2007, during which they will try to skip exon 51 and 46 by systemic application of the appropriate 2O-methyl AON.

These short-term trials will then be followed by long-term trials, lasting probably six months which could then possibly slow down the boys' Duchenne symptoms significantly.

The two exons to be skipped in the two first trials have been selected, because a successful skipping of exon 51 would be a therapy for up to 24% of all Duchenne boys with deletions, and skipping of exon 46 would help all boys with a deletion of exon 45, the most frequently deleted exon of Duchenne patients (8% of all deletions).

In addition to AONs for skipping exons 51 and 46, Prosensa has developed and also produced in sufficiently large quantities four other 2O-methyls. These six AONs would allow treating over 50% of all patients with deletions.

If all goes according to plan, it will take about four to five years, until the 2O-methyl AONs for skipping exon 51 and 46 will be ready to be marketed as a Duchenne medication. To fully develop additional AONs will hopefully be faster because the experience with the first two will likely considerably shorten the time for approval and testing the following ones.

Dr. Platenburg hopes to be able to report the results of this first exon skipping trial at the next Parent Project meeting in 2007.

**Exon skipping: Preparation of a clinical trial with Duchenne boys in the United Kingdom.** In the United Kingdom, the MDEX consortium was established to develop the exon skipping technique further and to perform clinical studies, thus to shorten the time as much as possible until a therapy becomes available for all Duchenne patients. With the first clinical study, the researchers will try to skip exon 51 of the dystrophin messenger-RNA in nine Duchenne boys. The members of the consortium are *Francesco Muntoni*, *Kate Bushby*, *Jenny Morgan*, *Dominic Wells*, *George Dickson*, *Ian Graham*, *Matthew Wood*, and *Jenny Versnel*, all of them are active in Duchenne research. The Department of Health and the Medical Research Council of the UK are also involved.

One of the MDEX members, **Kate Bushby** of the University of Newcastle upon Tyne, discussed the details of the upcoming trial and mentioned at the beginning that there will be close cooperation with several research groups outside the UK and especially with the Dutch group in Leiden. Many years of pre-clinical research has shown that *antisense oligoribonucleotides*, AONs, can "force" the mutated dystrophin gene to make a shorter protein, a Becker dystrophin, that would make the severe dystrophic symptoms of Duchenne boys much milder. Systemic applications of these AONs to living dystrophic mice practically "cured" these animals of their disease. And as AONs have already been used for years to combat other diseases, it is known that they are safe, not toxic. These positive results have convinced the British scientists to start at the end of this year a parallel clinical study to the studies already being performed in Leiden.

For the first MDEX trial, a number of decisions have already been made: The exon to be skipped will be exon 51, because many Duchenne mutations like the deletions 45-50, 47-50, 48-50, 49-50, 50, 52, 52-63, about 17% of all Duchenne deletions, could be treated by skipping of exon 51. The AON to be used will be the AON H51A, one of the *morpholinos* developed by *Steve Wilton's* laboratory in Perth in Australia against human exon 51. Nine Duchenne boys, 12 to 18 years old, will participate. Three different dosages: 0.09, 0.297, and 0.9 mg AON in 0.9 ml solution will be used, delivered into a volume of 1 cm<sup>3</sup> of muscle with nine injections directly into the muscle tissue. The target muscle will be one of the two *extensor digitorum brevis*, EDB, muscles on the outside of the foot that raises the toes. Humans do not really need it and many do not even have it. So it can be removed without serious consequences if some unacceptable side effects should occur. Extensive clinical checks including biopsies will be done before and five weeks after the injections on each boy as it is usual for clinical trials to assess the results of the treatment.

The main aims of the trial are to verify that the local administration of the morpholino AON into a single human muscle is safe and that it is effective to restore at least some dystrophin production. It is hoped that with the different dosages used dystrophin would appear in more than 10% of the muscle fibers. This would allow to get reliable results and also to estimate the total amount of AONs needed to treat all the muscles of a boy in a future systemic treatment.

The boys participating in this first trial with morpholinos injected locally will not get any therapeutic benefit. But all the results of this trial will be needed for a real treatment, for a systemic application of the potential Duchenne drugs into the blood circulation of the boy so that all his muscles can be reached. This second and more important trial is being planned for 2007.

**Exon skipping works in mice and dogs, but there are still many questions to answer before exon skipping is ready for the boys.** *Terence Partridge*, at present working at the Children's National Medical Center in Washington raised a number of questions which should be answered sooner or later before exon skipping can become an effective therapy for boys with Duchenne dystrophy. And he reported that in Japan exon skipping was shown to be working after local injection of AONs into dystrophic dog muscles.

We know that exon skipping in mdx mice works well with *morpholino* AONs against their exon 23 injected systemically into the tail vein. For instance, after seven weekly injections, the muscles look much better, they do not leak any more and therefore, the serum CK values become almost normal. But we do not know whether the AONs are evenly distributed in all muscles so that they all become better at the same time. We already know that the morpholinos unfortunately do not skip exon 23 in the heart muscles. Similarly, the other kind of AONs, the *2O-methyls*, also have some problem in affecting heart muscles. What is the reason for this failure?

Can we administer the AONs at sufficiently high concentrations that they are effective and non-toxic over the entire life time of a patient? We can do experiments on

tissue culture, but the same results cannot always be expected in living animals. Long-term experiments can be done in animals. But because dogs live much longer than mice and the dystrophic GRMD or CXMD golden retriever dogs are really physically handicapped, experiments with dogs would give results that would more likely be similar to what one would later see in clinical studies with Duchenne patients.

Such dog studies have now started in Japan at the Tokyo General Animal Research Facility under the direction of Professor *Shin'ichi Takeda*.

The dystrophic 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 sign soon afterwards. Skipping of the two flanking exons 6 and 8 would restore the reading frame. Different doses of a cocktail of three morpholino AONs, two different ones against exon 6 and one against exon 8, which were prepared by Dr. Toshifumi Yokota in Washington, were locally injected into the tibialis-anterior muscle of young adult CXMD dogs. Two weeks after the injection, biopsies were performed. When 1.2 mg of each AON dissolved in 1 ml of salt water were injected, new dystrophin appeared in all fibers of the muscle around the injection site and they looked almost normal. Systemic injections of the AONs will be the next experiments of the Japanese researchers who have "the largest dog house in the world" at their institution.

So morpholinos AONs work well in a large mammal with a quite similar body structure to humans, but this is not a guarantee that these potential drugs will work as well and over a sufficient long time in Duchenne boys. For this reason, other types of AON should be investigated, too. Dr. Partridge showed the structures of nine different types of AONs. Most of the work in the past years has been done with the morpholinos and the 2O-methyls, the two which have entered or will soon enter clinical trials on Duchenne boys. The two simultaneous trials now starting in the Netherlands and the UK are in fact very necessary and their high cost is justified, because a future exon skipping treatment might very well need a mixture of both types of AONs because, e.g. individual toxic effects of the two are likely to be independent of one another while their effects on exon skipping would be additive. And still unknown problems could appear at any time which one only would be able to solve with other AON cocktails, for instance, one of the other chemistries might work on the heart.

In addition, work will have to start and then to continue for a long time to select the most effective AON sequences, to determine the smallest dose that is still sufficiently active without being toxic or causing immune reactions, to find the most acceptable way of application, and finally to make the time between treatments as long as possible.

**Will exon skipping be able to produce new muscles in older Duchenne patients?** *Dominic Wells* answered: The muscle cells still present will be stabilized when new dystrophin is being made after exon skipping. Things would probably not get worse. Whether this would also re-establish function, one does not know. Possibly the combination of exon skipping with a pharmacological treatment, e.g., with an anti-myostatin drug, would be quite effective. Experiments in mice have been started.

## Transfer of the dystrophin gene.

The transfer, the transportation, of sufficient quantities of the intact dystrophin gene into the nuclei of dystrophic muscle cells would be an effective Duchenne therapy if the genetic information of the new genes is used by the protein synthesizing ribosomes of the cell to produce sufficiently large quantities of functional dystrophin which then migrates to its normal place under the cell membrane and connects correctly to the proteins of the dystrophin-glycoprotein complex.

The research teams of *Xiao Xiao*, now at the University of North Carolina in Chapel Hill and of *Jeffrey Chamberlain* at the University of Washington in Seattle are those working most actively in this field of gene therapy for Duchenne dystrophy. The scientists started their work by using viruses like the common-cold *adeno-viruses* as transport vehicles, as *gene vectors*. For this task, the viruses were modified in such a way that they cannot be multiplied by the cells they infect, because their genes for this multiplication were removed. In their place there is then room for the coding sequences of a therapeutic gene to be transferred together with certain control sequences. The sequence necessary for the production of the dystrophin protein, i.e. the *cDNA* of its gene, is 14,000 base pairs long, it contains all 79 exons connected to each other without the introns in between. The adeno-viruses are very effective in entering non-dividing muscle cells, and they do not deliver the transported genes into the genome of the target cells, they remain in the nucleus outside the chromosomes. This means, there is no risk of the arriving new genes being integrated at random into other genes or control sequences where they possibly could interfere with their activity or even cause cancer.

However, the most effective viruses for transferring dystrophin cDNAs into muscles are the *adeno-associated viruses*, AAV, which are about ten times smaller than the normal adeno viruses. But because they are so small, they can only transport genetic material that is not longer than about 5,000 base pairs, about one third of the entire dystrophin cDNA. Therefore, the normal dystrophin cDNA has to be shortened considerably to fit into this small vector. Patients with the benign Becker muscular dystrophy have such shortened dystrophins in their muscles. A transfer of such a *mini-gene cDNA* would not "cure" Duchenne muscular dystrophy but may instead transform it into something like the much slower progressing Becker form.

In order to determine which of the four domains or regions of the normal dystrophin protein – the two end regions, the cystein-rich, or the central rod regions – are important and which are not, the scientists created many different shortened dystrophin cDNAs and proteins. They identified some versions that were very functional and highly effective in living *mdx* mice. These artificial mini-dystrophins lack most of the central portion and the C-terminal end of the normal protein. The transfer of these selected mini-genes led to an improvement of muscle function and of all other dystrophic features of the *mdx* mice. In addition, the gene transfer showed better results in younger animals, and, after a single injection, the newly synthesized dystrophin remained in the muscles for one

year and longer.

**First clinical trial of dystrophin gene transfer.** *Scott McPhee*, Vice President of Clinical Development of the company **Asklepios Biopharmaceuticals** (Askbio) in Chapel Hill NC said that, 25 years ago, the founders of Askbio first started to work with adeno-associated virus, and based on their extensive experience with this gene vector they have now developed a biological nano-particle, BNP, trade-named *BioStrophin*<sup>™</sup> for the transfer of a mini-dystrophin to treat Duchenne dystrophy. One of their priorities is to fully pursue BioStrophin-based gene transfer as an effective therapy of Duchenne dystrophy. After extensive preclinical testing including toxicology and biodistribution studies with animals, and after the permission of the regulating agencies were obtained, the first clinical phase-I trial with six Duchenne boys has started. The purpose of this trial is to determine that the technique does not produce any toxic or other side effects, and to see if the mini-dystrophin is made properly in the muscles in human patients. This work is being supported by the Muscular Dystrophy Association of America with 1.6 million US\$ grant to Askbio. If the Phase-I trial indicates that the approach is safe and well-tolerated, additional funding will be sought for further clinical studies.

The vector used in this trial is a modified adeno-associated virus of serotype 2, called BNP2.5. It will contain a mini-dystrophin gene construction which does not contain parts of exon 17, all exons from 18 to 59 and from 70 to 79 inclusive. That means that the expected Becker dystrophin will be about one third as long as the normal protein because it lacks the rod regions R3 to R21 and the C terminal end.

This first phase-Ia trial is performed under the supervision of Dr. *Jerry Mendell* at the Children's Hospital of the School of Medicine at Ohio State University in Columbus. It has started on 28 March 2006, when the first boy received the first injections of BioStrophin at three sites, 0.5 cm apart, into his biceps muscle of one arm while the biceps of the other arm received only saline. The trial is done double-blind, neither the patients nor the medical and clinical investigators know until the end of the entire trial into which biceps the vectors were injected.

Six Duchenne boys who are at least five years old and whose mutations of the dystrophin gene are precisely known, are participating. Two different doses will be used for each group of three patients. The vector concentration at the local injection sites are much higher than in those used in the next trials when a systemic delivery into entire limbs will be attempted. Thus it will now be easier to see the results of the treatment and also any unexpected side effects like inflammation or immune reactions. If some serious problems should occur, it will be possible to remove the entire muscle tissue around the injection sites and thus stop the treatment prematurely and completely. After all, the effects of a gene transfer treatment of this kind will be long-term, therefore every step of the trials has to be done cautiously and with great care.

One and three months after the injections, tissue

samples from the injection sites will be removed by muscle biopsies. The samples will be kept frozen until the end of the entire trial when they will be checked for the presence of the new but shortened dystrophin.

The results of this phase-Ia trial will be available in about Spring 2007. There will be no therapeutic benefit for the participating boys. The next, so-called bridging or phase-Ib studies are now being prepared with dogs and monkeys. The bridging study will hopefully be performed in about 2008 and 2009. This time, a whole limb will get infusions of the vectors through the temporarily blocked blood circulation in a surgical procedure similar to what has been developed for delivery of chemotherapy agents. This trial of regional delivery may potentially offer some improvement in the quality of life of the participants. Finally, a phase-II/III trial with whole-body systemic delivery is planned for 2009/2010 with a larger number of patients than the early phase trials. If this is successful, the further progression of the Duchenne dystrophy of the boys may already be prevented in what is hopefully a one time procedure.

**The promise of stem cells.** In his second presentation, *Terence Partridge* discussed the rather unfulfilled promise of stem cell research to deliver a therapy for Duchenne muscular dystrophy - with one exception.

Stem cells can divide for indefinite periods of time and they have the ability at each cell division to give rise both to a similar stem cell as well as to a more specialized cell type. They thus can undergo *asymmetric cell division*, to maintain the population of themselves more or less constant and to assure that more specialized cells can continually be repaired throughout the life of an organism.

There are different kinds of human stem cells: The first eight cells of a human embryo are *totipotent stem cells*, because every one of them can develop into every type of cell of the body. Four to five days after fertilization, the interior cells have formed the inner cell mass of the blastocyst, these cells are called pluripotent *stem cells* or *embryonic stem cells*. They interact with each other and produce *lineage-restricted stem cells*, committed or *adult stem cells* each of which can only form one or a few of the specialized tissues of the body. The adult stem cells for the formation and repair of muscle cells are the *satellite cells*.

If muscle tissue is damaged, the satellite cells, which reside on the outside of the muscle cell, move into the damaged area, divide and fuse to myotubes which then develop further to mature muscle cells. Some of the satellite cells in the new muscle tissue divide asymmetrically, they produce also new satellite cells which then take up their normal place on the outside of the repaired cells to be ready when they are needed for the next round of regeneration of muscle cells, which were, e.g., degraded by dystrophic processes.

Satellite cells can also be injected, locally grafted, into damaged muscles where they behave like myogenic stem cells for the repair of damaged muscle tissue. These cells can act very fast, in a few days, a few of them can produce many thousand of muscle cell nuclei which then form entire muscle fibers. If these external satellite cells come from normal muscle with an intact dystrophin gene, the

new muscle then also contains dystrophin even when, as in experiments with mdx mice, the surrounding muscle tissue cannot make it.

But for a Duchenne therapy, the local administration in all muscles with a huge number of injections, would not be an acceptable therapeutic procedure. The possibility of a systemic administration with a few injections into the blood circulation would be needed so that all muscles, even those of the heart and the lungs, could be reached.

Thus, for an effective stem cell therapy of muscular dystrophy, a safe and ethically acceptable source of a large amount of adult muscle stem cells is needed, that would give rise exclusively to muscle cells and to nothing else, especially not to tumors. And it should be possible to apply these cells systemically by injecting them into the blood vessel system which could distribute them around the body. Then they have to cross the membranes of the muscle cells, and stay there without creating any local trouble. However, with one exception, no source of stem cells has been found which would meet all these conditions in experiments with living mdx mice.

The exception mentioned are *mesoangioblasts*, which are adult stem cells located on the outside of small blood vessels within the muscle tissue. *Giulio Cossu* and his colleagues at the Stem Cell Research Institute of the University of Pavia in Italy recently performed a basic experiment whose results will become very important for a stem cell treatment of many different muscular dystrophies.

They did not use the mdx mouse as an animal model but a mouse whose gene for alpha-sarcoglycan, one of the proteins of the dystrophin associated complex, was made inactive. These mice have a type of limb girdle muscular dystrophy, LGMD, which is clinically somewhat similar to Duchenne dystrophy. The Italian researchers isolated mesoangioblasts from normal mice, treated them with several different growth factors and then injected them into the blood circulation of the LGMD mice. These "healthy" stem cells were able to migrate into all skeletal muscles of the living mice and caused the re-appearance of more than 80% of the normal amount of alpha-sarcoglycan.

For a possible Duchenne therapy using this new technique, intact dystrophin genes would have to be transferred into patient-derived mesoangioblasts by an ex-vivo procedure with known vectors, then multiplied in the laboratory, and finally re-injected into the blood circulation of the patient. Possibly, this treatment would have to be repeated periodically, therefore it is important that these cells would not be regarded by the immune system as 'non-self' and rejected.

At present, this approach is the most promising example of stem cells as a therapy for Duchenne muscular dystrophy. All other stem cells tried in living mice did not give impressive results. The problem may lie in the muscle tissue itself rather than in the stem cells, so the scientists have to find the reason for these difficulties.

Dr. Partridge finished his talk with a warning that there are many claims of different stem cells as potential therapies for Duchenne dystrophy. In most cases, these claims are not well founded. Muscle biology is full of traps for the unwary and sometimes even experienced scientists can be misled by what they believe they are seeing.

## Pharmacological approaches.

**Reading through premature stop codons.** Proteins are synthesized on *ribosomes*, complex structures consisting of three large RNAs with enzymatic activity, *ribozymes*, and about 80 different proteins. The information for the production of proteins is brought to the ribosomes as mRNAs. Inside the ribosomes, the new protein is assembled from its building blocks, 20 different amino acids. They are delivered to the assembly site by another kind of RNA, the transfer or tRNAs, which recognize the triplet codons of the mRNA one after the other. When a normal stop codon arrives at the synthesis site, meaning that the protein is now ready to be completed, special proteins, *release factors*, enter the assembly site, bring the synthesis to a halt and release the completed new protein from the ribosome.

About 10% to 15% of Duchenne boys have a point mutation in their dystrophin gene which changes an amino acid codon into one of the three stop codons, TGA, TAG and TAA. In the mRNA, these codons become UGA, UAG, and UAA and cause protein synthesis to shut down prematurely, before the new protein, in this case dystrophin, is fully assembled.

The antibiotic *gentamicin* has been shown to interfere with the mRNA translation mechanism in the ribosomes so that it ignores such a premature stop codon, i.e. it *reads through the stop codon*. In animal models with a premature codon in the dystrophin mRNA, treatment with gentamicin has been shown to induce read-through and has partially restored production of full-length, functional dystrophin protein. However, gentamicin can be toxic and must be taken intravenously, so its long-term use as treatment for Duchenne muscular dystrophy is not practical.

If an improved read-through technique can be developed as a therapy for Duchenne muscular dystrophy, the subset of patients with a nonsense mutation as the basis for the disease may be able to benefit from it. Based on the pre-clinical studies in mice, the amount of new dystrophin made, will not cure the disease but may change it to a Becker-type dystrophy. As read-through does not occur at the gene level but during protein synthesis in the ribosomes, the treatment will have to be taken daily. To determine whether a Duchenne boy can be treated with a read-through drug, it should be known that he has a point mutation in his dystrophin gene and that it has introduced one of the three different premature stop codons.

**PTC 124:** *Langdon Miller*, Chief Medical Officer of the company **PTC Therapeutics** in South Plainfield NJ reported on the development and clinical testing of *PTC124*, a new chemical compound that is much more effective than gentamicin at reading through premature stop codons. Finding a therapy for Duchenne muscular dystrophy and cystic fibrosis, based on stop codon read-through, are two of PTC's priorities.

Several thousand chemical compounds were automatically tested for their ability to read-through premature stop codons in the mRNA for dystrophin or for the CFTR protein, the protein that is absent in cystic fibrosis. The structures of the most promising compounds were then changed through many chemical variations until one was obtained, PTC124, that is more efficient than gentamicin.

In the Duchenne project, full-length dystrophin appeared in almost normal amounts in cell culture or in up to 25% of the muscle fibers of living mdx mice after oral application with a substantial rescue of their function and tissue structure. No read-through of normal stop codons was detected. Toxicity studies in rats and dogs given high doses of the drug have generally not shown serious, acute side effects. This potential Duchenne drug is a powder that can be made into tablets and taken by mouth.

In the first clinical testing of PTC124, two phase-I trials were performed in adult healthy volunteers. In these studies, the drug appeared safe and demonstrated few side effects. Last year a phase-II trial of PTC124 in 15 cystic fibrosis patients has been performed. It showed that PTC124 is capable of partially restoring CFTR activity in these patients and did not show any serious adverse effects.

At three clinical centers in the United States, a phase-II trial to test the effect of PTC124 in Duchenne patients is also now in progress. Twenty-two 5-12-year-old Duchenne boys with premature stop codons in the dystrophin gene participate in this study. The study was designed to last eight weeks. During the study, six boys were given a lower dose of PTC124 three times per day for four weeks followed by a four-week follow-up period without medication. When there were no adverse effects another group of 16 boys received a higher dose of PTC124 for four weeks, again followed by four weeks without the drug. To assess the outcome of the trial, muscle biopsies were performed before and after the treatment course to evaluate for the partial restoration of full-length dystrophin production. Other chemical and functional tests were also done to quantitatively measure the therapeutic effect of PTC124 in Duchenne boys. These results will be available at the end of 2006. If they are as positive as expected from the pre-clinical development phase, and if the necessary permission is obtained from the regulatory agencies in Europe and the United States, a phase-III trial will possibly start next year or in 2008.

**Upregulation of utrophin.** Utrophin is a protein with a structure and function very similar to dystrophin. In humans, its gene is located on chromosome 6, it has 75 exons and is about one million base pairs long. The utrophin protein is about 7% shorter than dystrophin. It is present in many body tissues, also in muscle, but there it is concentrated in regions where the motor nerves contact the muscle membrane, the *neuromuscular junctions*. Utrophin exists in two slightly different forms A and B. The muscles contain only the A-form. Before birth, the utrophin concentration in muscle is much higher than afterwards. Mdx mice whose utrophin gene was *knocked out* experimentally, which thus have neither dystrophin nor utrophin, have Duchenne-like symptoms and die early in contrast to "normal" mdx mice whose muscles show less severe damage.

Experiments with mice have shown, that utrophin, if it is present in larger amounts, can replace dystrophin. These mice were transgenic mice who contained utrophin mini genes in their germ line, introduced by a technique that cannot be used in humans. By increasing the amount of utrophin by a factor of three to four, the development of

the dystrophic symptoms could be prevented and led to a complete functional recovery.

It has recently been found that Duchenne boys who have slightly higher amounts of utrophin in their muscles lose their walking ability later than those with the normal low amount. This is an indication that increasing utrophin would work like in mice and prevent or retard the degradation of the muscles.

For a possible Duchenne therapy, one should try to increase the low amount of utrophin by *upregulation* of the activity of its gene. To achieve this, an activating substance is needed, which could well be a known drug, or some other chemical or a naturally occurring substance, that would react with the promoter of the gene. The small molecules of such a compound could probably enter the muscle cells easily, and if they are known drugs, they would not need lengthy approval procedures.

**Kay Davies** of Oxford University, who with her team has pioneered research on utrophin as a replacement of dystrophin for many years, reported that in cooperation with the company **VASTox plc.** in Oxford thousands of chemical compounds were screened for their ability to upregulate the activity of the utrophin gene in mdx mice. The light producing enzyme luciferase from fireflies was used in a reagent system to test for this activity. Several promising candidates were found which are now being optimized and tested on muscle cell cultures and in living mdx mice, to see whether they can sufficiently increase the amount of utrophin in all muscle of the animals.

One of the most promising active compounds has already been tested systemically in mice by injection into the abdomen. It reacts only with the promoter of the A-form of utrophin, that is present in muscle. The A-utrophin in all of the skeletal muscles of the mice tested could be upregulated two to three fold, but it is not known yet, whether the utrophin in cardiac muscles is upregulated also. After 12 weeks of weekly injections, the animals showed a significant recovery of their muscular function.

This active compound is now being optimized further by additional chemical modifications. Clinical trials with Duchenne patients are being prepared and could start in 2008.

**Inhibition of proteases.** The degradation of muscle proteins in Duchenne dystrophy is caused by several different proteases, protein-destroying enzymes, such as the enzyme *calpain*, which is activated by calcium, and a large protease complex, called the *proteasome*. When, as in Duchenne dystrophy, muscle cell membranes become leaky because dystrophin is absent, calcium ions, charged atoms, from outside of the cells activate calpain and indirectly also the proteasome. This increased enzymatic activity leads to widespread destruction of important cellular proteins that are required for muscle cell function and survival. Specially designed inhibitors allow researchers to block the activity of calpain and other proteases which can delay muscle cell degradation. The modified tripeptide *leupeptin* was the first inhibitor identified that could reduce calpain activity in mdx mice. This first-generation inhibitor consists of three amino acids, two leucines and one arginine, with the arginine containing a chemically reactive aldehyde group that is essential for the inhibitory

activity. Leupeptin, however, can also inhibit other proteases including those of the blood plasma coagulation cascade, causing intolerable side effects. On the other hand it might be desirable to inhibit not only the calpain enzyme but also the proteasome.

**C101. Theresa Michele**, Vice President of Clinical Research of the **CepTor Corporation** in Baltimore described that by combining leupeptin with carnitine, an inhibitor, called C101, was obtained which only enters skeletal and heart muscle cells. The reason for this specificity is that there exists a protein, the carnitine transporter OCTN2, which binds C101 at its carnitine end and carries it to a receptor protein on the muscle cell membrane, which then transports the inhibitor C101 across the membrane into the cells.

In order to measure precisely the effect of a potential drug like C101, a new quantitative test method had to be developed that allowed analysis of the calpain-induced breakdown of muscle cell proteins in living animals and possibly in humans, too. In this test, the ability of calpain to cleave the muscle cell protein alpha-II-spectrin is measured. Calpain uses two unique steps to break the spectrin protein down to the two smaller proteins, SBDP150 and 145, which leak out into the blood and then can be analyzed in serum with specially designed antibodies. This new test is designed to follow the progress of muscle degradation and thus will probably become important for clinical trials with Duchenne patients.

With this new test, it was shown that C101 can inhibit calpain 50 to 100 times more effectively than leupeptin. It also preserves the structure of the muscles and increases the diameter of the muscle fibers in mdx mice significantly. C101, which can be administered orally, thus is a potentially effective drug for treating Duchenne patients.

**BBIC.** In his overview of the different therapeutic approaches, **Lee Sweeney** mentioned the *Bowman-Birk inhibitor concentrate*, BBIC, that blocks other proteases than calpain which also participate in the destruction of muscular proteins. The active substance in this raw concentrate is a natural protein composed of 71 amino acids that can be isolated in pure form from soybeans.

Long-term treatment with BBIC increases the muscle mass and strength in mdx mice. CK activities are reduced considerably and fibrosis also. And from other applications in cancer patients it is known that BBIC is a very safe drug which can be applied orally.

**SNT 198'438. Thomas Meier**, Chief Scientific Officer at **Santhera Pharmaceuticals** in Liestal near Basel in Switzerland, described their preclinical studies for the development of a dual-specificity inhibitor which simultaneously can block the activity of calpain and the proteasome enzyme complexes. Starting with a known calpain inhibitor scientists at Santhera have synthesized over 800 chemical variants and tested these inhibitors in biochemical and cell culture experiments as well as in mdx mice. Several compounds with the desired properties were identified.

One of them, SNT 198'438 was further optimized: It can be administered subcutaneously, i.e., by injection under the skin. Thus, it acts systemically and reaches and enters all muscles. In mdx mice, where it is well tolerated,

this inhibitor normalizes histological parameters of the muscles and improves the exercise performance of adult animals. This function test is performed in a large "mouse gym" where up to 40 mice can run voluntarily for several weeks in computer-monitored running wheels.

**SNT-MC17/idebenone to protect mitochondria.** **Santhera Pharmaceuticals** is currently also developing a potential drug that protects mitochondria, the power stations in the cells where the universal energy carrier, adenosine triphosphate, ATP, is made by oxidative phosphorylation. **Thomas Meier**, in the second part of his presentation, explained that this compound, *idebenone*, or SNT-MC17, is now in a phase-III clinical trial for Friedreich's Ataxia in the United States and Europe. Friedreich's Ataxia is a neuromuscular disease which frequently is associated with cardiomyopathy, a severe disease of the heart muscle.

SNT-MC17/idebenone is a potent antioxidant with a chemical structure derived from natural coenzyme Q10. The optimized chemical structure has a much shorter and different side chain which allows the molecule to enter muscle cells easier than coenzyme Q10. SNT-MC17/idebenone has also been shown to facilitate the ATP production in the mitochondria. It can be given orally as a tablet.

The absence of dystrophin also negatively affects the oxidative phosphorylation in the mitochondria of the heart muscles of Duchenne patients and probably in those of their skeletal muscles, too. A phase-IIa double-blind, placebo-controlled clinical trial with SNT-MC17/idebenone is currently underway in Belgium under the leadership of Dr. **Gunnar Buyse**. The study has completely enrolled 21 Duchenne boys at 8 to 16 years of age. The primary objective of this study is to determine the effect of SNT-MC17/idebenone on heart muscle function. In addition several different tests will be performed to detect the possible functional benefit on muscle strength in Duchenne boys treated with SNT-MC17/idebenone. The boys are receiving the study medication three times a day in form of tablets containing either 150 mg SNT-MC17/idebenone or placebo for 12 months.

This trial is called "Duchenne Efficacy Study in Long-term Protocol of High dose Idebenone", abbreviated DELPHI. Its results will be available in about one year.

An interview with Dr. Meier can be seen on the Internet at [www.duchenne-research.com](http://www.duchenne-research.com).

**Poloxamer 188, a "molecular band-aid" to close holes in membranes.** An attempt to rescue heart muscle cells in cardiomyopathy opens a way to close the holes in the membranes of dystrophic muscle membranes. This new approach to a Duchenne therapy was reported by **Joseph Metzger** of the University of Michigan in Ann Arbor. The absence of dystrophin in mdx mice as well as in Duchenne boys not only affects skeletal muscles but has also severe consequences for the correct function of cardiac muscles.

The use of a specially designed polymer, the *poloxamer 188* or P 188, had been shown to influence positively the heart function of mdx mice. To exactly measure this protective effect in isolated mdx heart muscle cells, single cells were connected with their ends to two very thin movable carbon fibers. As these cardiac muscles are about thousand times shorter than the skeletal muscle cells of

mice, the distance between the two micro carbon fibers can only be varied between 1.8 and 2.2  $\mu\text{m}$ , thousandths of a millimeter. In this way, it could be shown that, whereas normal cells could be stretched and relaxed for many hours, mdx fibers have a reduced cellular compliance, they are more resistant to extension, get into a hypercontraction, break away from the carbon fibers and finally die. With this completely new *microscopic test method*, it could be shown that P 188 can restore the compliance of the cardiac muscle cells from mdx mice and also from the dystrophic GRMD dog. In-vivo application of P 188 to living mdx mice blocks their acute heart failure which could be measured with mini catheters in the hearts of living mice beating about 600 times a minute.

P 188 is an artificial co-polymer, it consists of a core of 35 small hydrophobic, water repellent, units and two wings of 75 units each of a hydrophilic, water attracting structure. Dr. Metzger called this a molecular *albatross*, whose central water-insoluble structure plugs the holes of the membrane whose interior is also hydrophobic, while the two hydrophilic wings act like the sticky ends of a band-aid by binding to the hydrophilic surface of the intact membrane around the holes. This molecular band-aid thus seals the holes at least temporarily so that no calcium ions can pass which would activate calpain and other protein-degrading enzymes.

It is obvious, that this protective effect of poloxamer 188 on cardiac muscles could also be important for the repair of dystrophic skeletal muscle cells. This has been tried, however the first results were not as good as expected. Research on this approach is now in progress and could lead to another possibility for a Duchenne therapy.

**Inhibition of myostatin:** *Myostatin* is produced in muscle cells as an inactive protein consisting of 375 amino acids. After several steps of molecular rearrangements, it becomes biologically active. and then initiates a series of chemical reactions inside the cell, which lead to the down-regulation of enzymes for the biosynthesis of new muscle proteins. Therefore by inactivating myostatin, the regeneration of the muscle fibers of Duchenne boys could possibly be stimulated so that they would not be destroyed as fast or might even increase in size.

Non-dystrophic mice whose gene for myostatin had been knocked out by genetic methods, have up to three times larger skeletal muscles with significantly more fibers of larger than normal diameter. There are cattle, the Belgian Blue Breed, which are very muscular because their myostatin gene was inactivated by a mutation centuries ago. And in Berlin, a now 7-year old boy was identified whose skeletal muscles are about twice as large as in a normal child. He is physically very strong. His mother was an Olympic runner, and several other relatives were also very strong. Because of a mutation in this family had changed the normal splicing of the three myostatin exons, the boy and probably his affected relatives, too, have a very low level of myostatin in their muscles. This is a strong indication that downregulation of myostatin would lead to an increase of muscle growth in Duchenne boys, too.

**Myo 029.** **Kathryn Wagner** of the Wellstone Muscular Dystrophy Center at the Johns Hopkins University in

Baltimore reported that her research team had raised mdx mice which, in addition of not having dystrophin, also could not make any myostatin. Adult mice of these myostatin knock-out animals had more normal muscles, had less fibrosis, scar tissue, and they regenerated their muscles faster than "normal" mdx mice. Together with Dr. *Lee Sweeney*, similar experiments will be performed on dystrophic dogs.

The question was now whether the absence of myostatin would have similar effects on the heart. This would counteract a cardiomyopathy, but a hypertrophic, an enlarged, heart would be problematic in Duchenne boys. However, recent investigations with mdx mice showed that the blockade of myostatin had no effect on the heart. This means that the activity of myostatin seems to be restricted to skeletal muscles alone.

In cooperation with the company **Wyeth Pharmaceuticals**, a clinical phase I/II trial with three different dosages of the potential drug Myo 029 was started with 36 adult muscular dystrophy patients, inclusive some Becker patients. Myo 029 is a specific antibody which binds to myostatin and blocks its activity. It does not cause immune rejection because its protein structure is the human one, it is "humanized". It can be injected into the circulation or under the skin.

If the trial should give encouraging results, Wyeth will intensify their efforts to bring Myo 029 to the clinic. In the meantime, parents should not buy any so-called myostatin inhibitors offered on the Internet. These compounds have not gone through clinical trials and therefore are probably ineffective or even dangerous.

**Upregulation of insulin-like growth factor, IGF-I.** IGF-I is a protein with about 70 amino acids in one chain with three stabilizing bridges, thus with a similar shape as insulin. Six different forms can be produced in humans with slightly different structures, but resulting in the same IGF-I protein. IGF-I is very beneficial for muscle, because it helps to promote growth and strength. However, the effects of IGF-I are not limited to muscle. The satellite cells, when activated by injury or degradation, produce a specific receptor protein in their membranes, to which IGF-I binds. The consequence is a stimulation of the proliferation of the satellite cells and their further development to myotubes and muscle fibers. As this stimulated regeneration of muscle fibers would be important for maintaining dystrophic muscle tissues, IGF-I is of interest for a possible therapeutic use in Duchenne children. However, other tissues can also respond to IGF-I, and when there are high levels in the blood, there is increased risk of cancer. Therefore, in order to establish IGF-I as a therapy for muscle disease, strategies must be developed to reduce the potential side effects in other tissues.

The research team of *Elisabeth Barton* of the University of Pennsylvania in Philadelphia works with mice which were obtained by crossing mdx mice with transgenic, i.e. genetically engineered, mice that produce high levels of the 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 usual mdx mice. This work demonstrates the benefits the IGF-I could have for Duchenne children.

But because this growth factor interferes with many signalling pathways in cells, potentially serious side effects cannot be excluded if higher dosages are used to optimize the effect on muscles. For this reason, a method was developed to "mask" the IGF-I by complexing it with the IGF binding protein-3 which is a naturally circulating protein in the bloodstream. This complex releases IGF-I only where and when it is needed, and helps to stabilize the protein in the circulation so that fewer injections are needed. A commercial formulation of this complex, called IPLEX™ is already approved by the FDA for the treatment of growth failure in children due to IGF-I deficiency. A first clinical trial with IPLEX is now being performed at University of Rochester with support from the NIH and MDA with 15 adult myotonic dystrophy patients. A trial to optimize the dosage will follow in 2007. 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 transport its gene into the muscles by a vector like the adeno-associated virus (AAV) which would then instruct the muscles to make more IGF-I. First experiments in that direction have been done in Dr. Barton's laboratory which showed that only one of the two similar forms of IGF-I, namely IGF-IA, is effective in mdx mice at promoting hypertrophy, the enlargement of muscle fibers. Current work with this technique succeeded in increasing the level of IGF-I 30 to 40 fold after intramuscular injection of the AAV vectors carrying the correct IGF-I gene. The newly synthesized IGF-I stayed in the muscle tissue, it did not leak out into the blood, thus side effects caused by activation of non-muscular tissues may be avoided. Viral gene therapy will take several years until it could be tried in Duchenne boys. However, this research will help to identify which form of IGF-I works best for muscular dystrophy.

**Project Catalyst: Looking automatically for new drugs.** *Ellen Welch*, a research group leader at **PTC Therapeutics** in South Plainfield NJ introduced *Project Catalyst*, which was started two years ago and which is supported by our Parent Project. This is an example of how a dedicated small company uses the most modern automated techniques to find among about 200,000 small-molecular weight compounds the very few that could possibly be candidates for drugs that would do what was discussed in the previous three paragraphs: upregulation of utrophin, downregulation of myostatin, upregulation of the muscle-specific isoform of IGF-1, and, in addition, upregulation of alpha-7-integrin.

Experience during the development of PTC124 for stop-codon read-through helped to optimize the automatic screening methods to modulate the activity of all four drug target molecules. The test procedure to measure the activities is based on a reporter protein system: the different regulatory sequences found at the ends of the target mRNAs (known as the untranslated regions [UTRs]) for each of the four targets were combined with the gene for the enzyme luciferase that normally produces light in fireflies. This firefly construct was introduced into kidney cells in such a way that in the presence of an active compound, the light intensity of the luciferase reporter would be either increased or decreased. The vast majority

of inactive compounds would not change significantly the light intensity. Measuring precisely and automatically light intensities in a very small volume of a sample preparation is much easier than analyzing the biological effect of the targets utrophin, myostatin, IGF-1, and alpha-7-integrin.

Two high-throughput screens were performed for each of the four targets. Among the 200,000 tested compounds several could be identified that either upregulated utrophin, IGF-1, or alpha-7-integrin, or that downregulated myostatin. All these "hits", compounds with at least some of the desired properties, are now being optimized by varying their structure. This will occupy many chemists for several years. E.g., for the optimization of the read-through drug PTC 124, about 4,000 chemical modifications were made to the originally identified active structure during two years of laboratory work.

The next steps in the preclinical development of the new potential Duchenne drugs will also take several years. In addition to the structural changes, there will be a detailed investigation of the biological properties of the most active molecules including studies in cell culture and in living mice of possible toxicity, of the metabolism, and of the pharmacokinetic behavior, i.e., of the biochemical changes inside a living organism which might produce, but hopefully not, undesirable side effects.

The clinical testing in Duchenne patients of the most promising substances will then follow and will require a few more years.

**Inhibition of TGF-beta.** In the first part of his presentation, *Andrew Hoey* of the University of Southern Queensland in Australia explained the role of fibrosis and what possibly could be done to prevent or to reduce it.

Fibrosis, or scar tissue, is caused by the excessive production of connective tissue and its deposition between the skeletal and cardiac muscle fibers 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 from cells, called fibroblasts. This occurs during the degeneration and regeneration process in Duchenne Muscular dystrophy under the influence of growth factors, among them *transforming growth factor beta*, TGF-beta.

TGF-beta promotes the synthesis and merger of different collagen molecules to produce the highly inflexible connective tissue. Thus inhibiting the activity of TGF-beta may be a possible mechanism to reduce fibrosis. One such drug that may have potential is *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 TGF-beta 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.

**L-arginine and nNOS.** In the second part of his presentation, *Andrew Hoey* discussed another consequence of the missing dystrophin, the reduced amount of one particular component of the dystrophin-glycoprotein complex, the enzyme *neuronal nitric oxide synthase*, nNOS. This en-

zyme 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 be the cause of increased fibrosis in hearts of *mdx* mice and also of Duchenne patients.

Daily administration of L-arginine for 6 months commencing in 6-month old *mdx* mice 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.

**Blocking inflammatory agents.** The degradation and death of muscle cells causes inflammatory processes which 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. They are being widely used in Duchenne boys to maintain muscle function for at least a few years. But their exact mechanism of action is still not well known.

*Sylvia Lopez* a graduate student in the Laboratory of Dr. *Melissa Spencer* at the University in Los Angeles reported on new experiments to counteract inflammation and immune response and thus to find new ways to eventually replace the steroids with drugs that target specific immune mediated damage.

Studies have shown that increased levels of the CD4 and CD8 T cells of the immune system accelerate the progression of the disease and that their inhibition reduces the rather slight dystrophic symptoms of *mdx* mice and very significantly the much more severe symptoms of the really sick *utr-/mdx* mice which in addition to the missing dystrophin also do not have any utrophin. This also extends the life span of these utrophin-minus *mdx* mice.

In addition, the amount of cytokines, molecules that promote inflammation and the development of fibrosis, is increased in *mdx* and Duchenne muscles. Therefore, inhibition or removal of CD4 and CD8 T cells and also the modulation of active cytokines would possibly slow down the degradation of dystrophic muscles. A number of approved or potential anti-inflammatory drugs already exist. If they could be shown to positively influence Duchenne dystrophy, the time for the additional approval for the treatment of DMD could be considerably shortened.

Three of the drugs are now being tested in Dr. Spencer's laboratory to see whether they would be beneficial for Duchenne patients. They are already being tested in clinical trials for other diseases: *CTLA-4Ig* against rheumatoid arthritis, *Galectin-1* is being lobbied and pushed into clinical trials for arthritis and has already been shown to improve muscle regeneration, and *Anti-asialo GMI* an antibody being used in Parkinson's disease - just to name some of the target diseases.

Testing of four other drugs will follow: *Raptiva*®, approved for psoriasis; *Tisabri*®, approved for multiple sclerosis and Crohn's disease; *Remicade*® and *Enbrel*®,

both approved for rheumatoid arthritis and other diseases.

Long-term treatment studies will be necessary to establish whether these drugs could become therapies for Duchenne dystrophy and thus be able to improve the quality of life for Duchenne patients.

**Biglycan.** At the end of the conference as "breaking news", **Justin Fallon** of Brown University in Providence RI described an unexpected discovery that could lead to a therapy for Duchenne Muscular Dystrophy. His laboratory has been working for the past eight years on the extracellular protein *biglycan*, BGN. This until now rather unknown protein connects the two ends of the proteins alpha- and gamma-sarcoglycan of the complex on the outside of the membranes of skeletal muscles. In experiments with mice whose gene for BGN had been eliminated, it was found that the amount of many proteins of the dystrophin complex was reduced. Treating these mice with local and systemic injections of BGN led to the re-appearance of beta-

syntrophin, which was an indication that the dystrophin complex was restored. Biglycan seems to be particularly important in juvenile mice at a time when the muscle do not need dystrophin, but rather rely on utrophin.

Very recent work presented at the meeting showed that biglycan can also be administered to mdx mice where it had positive effects on countering the dystrophic pathology of the muscle. Since BGN is normally present in low concentrations in mice, immunological reactions did not appear, and none would be expected in humans either. Alpha- and gamma-sarcoglycans are only present in skeletal and cardiac muscles. And because BGN binds to these two proteins, it could be active primarily in these two types of muscles, and thus may have minimal side effects.

Experiments with animals will continue to optimize treatment conditions. Phase-I clinical trials could then be started in two years with this unexpected potential new Duchenne drug: *biglycan*.

### Why is the mdx mouse not really sick?

**GAMT.** The question was being asked by **Brian Tseng** and his research team at the University of Colorado in Denver. The mdx mouse has no dystrophin protein due to a premature stop codon in exon 23 of its dystrophin gene, however it does not show crippled clinical features of human Duchenne muscular dystrophy, it is remarkably healthy! For instance, it runs about 5-6 km per night every night in voluntary mouse running wheels. It lives nearly two years, as long as normal mice. If observed side by side an mdx mouse cannot be distinguished from a normal mouse. The mdx mouse does show some laboratory features of the Duchenne disease, e.g.: high serum CK levels; centrally located cell nuclei; slightly increased fibrosis, and a rather severely affected diaphragm although respiratory failure is not seen.

Could the mdx mouse teach us how to develop treatment strategies for a more benign form of dystrophin-deficient muscular dystrophy? Dr. Tseng believes there are *modifier genes* that are upregulated in the mdx mouse, but downregulated in Duchenne boys. Upregulation of utrophin, satellite cells, revertant fibers, and downregulation of myostatin are effects of modifier gene that are expressed in parallel in both mdx mice and boys with Duchenne dystrophy. Dr. Tseng is most interested in studying the genes that are expressed in opposite directions in mdx mice versus Duchenne boys. As there are probably other modifier genes that cause the different disease symptoms, Dr. Tseng and his colleagues looked with large-scale automatic screening techniques at the mRNA levels of 30,000 genes of skeletal muscle from adult mdx mouse compared to normal mice.

They found 45 upregulated genes which, in contrast, were downregulated in Duchenne boys. Two of them

looked very promising: the genes for the enzymes *Arginine:glycine amidotransferase*, AGAT, and *guanidinoacetate methyltransferase*, GAMT, which are necessary for the biosynthesis of creatine, a low-molecular compound that plays an important role for the supply of chemical energy for muscle contraction and cell health. In the mdx mouse, both enzymes are upregulated, so the mdx mouse can make creatine in its muscles. Dr. Tseng believes that the absence of dystrophin in Duchenne boys and in mdx mice causes problems with another protein, the creatine transporter in the muscle membranes, so oral creatine does not work well. It seems that the upregulation of AGAT and GAMT and the mislocalization of the creatine transporter protein are all secondary consequences of the absence of dystrophin in the mdx mice. Therefore, an mdx mouse was created whose GAMT gene was inactivated. This *double-null mouse* cannot walk properly, it dies early and its muscle structures look similar to those from biopsies of Duchenne boys.

Furthermore, Duchenne boys have only 20% of the normal amount of creatine in their muscles, whereas in mdx mice, 80-90% of the normal level of creatine is present. This can be one of the reasons for the much more severe dystrophic symptoms of the boys compared to those of the mdx mouse. Further investigations of these and other differences may help explain why the mdx mouse is not really sick, and this may uncover new treatments for Duchenne muscular dystrophy. One direction to pursue is high throughput screening, perhaps in *Project Catalyst* for these modifier genes! Turning on "good modifier" genes *safely* may be a reasonable goal to achieve a more benign muscular dystrophy for Duchenne boys.

### If there is still no cure, why do we need to know the exact mutation?

**Kevin Flanigan** of the University of Utah in Salt Lake City gave the answer: The exact mutation of a boy who seems to have Duchenne dystrophy should be known to confirm that he really has Duchenne dystrophy, and not

some other muscle disease, e.g. one of the many limb girdle dystrophies which might show symptoms similar to Duchenne. Although the type of mutation does not always predict Duchenne versus Becker muscular dystrophy, if the

mutation shows that the dystrophin reading frame is shifted, Duchenne muscular dystrophy is much more likely. In addition, in most cases, a muscle biopsy can be avoided. Equally important, knowing the exact mutation allows reliable genetic counselling of the boy's family and his maternal relatives, among whom Duchenne carriers can be detected. Finally, new therapies, such as exon-skipping methods, and stop-codon readthrough drugs require knowledge of the specific mutation within the patient's dystrophin gene.

In order to detect deletions and duplications, the analytical technique now widely used is the *multiplex ligation-dependent probe amplification* method, MLPA, developed a few years ago by Dr. *Jan Schouten* of the company *MRC-Holland* in Amsterdam. To give a very short description of the procedure: 158 oligodesoxynucleotides with specially designed sequences to bind at two sites of each of the 79 dystrophin exons are used. If an exon is present, the two nucleotides designed for its particular sequence bind on two sites and then are connected, ligated, to each other. The ligated nucleotides serve as a template for a PCR amplification, a multiplication method. The amplified product can then be seen after electrophoretic separation as a peak on a chart. If a particular exon is not present because it is deleted, the two nucleotides for this exon cannot bind to the exon sequence and thus cannot join each other, so the corresponding peak is missing on the chart.

This technique detects deletions and duplications of all 79 exons of the dystrophin gene in Duchenne patients, but does not detect point mutations. But because it is a quantitative method, deletions and duplications can also be reliably detected in just one of the two dystrophin genes of Duchenne carrier women, even if the deletion or duplication in the related patient is unknown. This is one of the most important advantages of the widespread use of this method.

However, false positive results, that indicate a deletion of a single exon when there is none, may occur in the rare event of a polymorphism at the site where the MLPA probes bind to the gene sequence. A polymorphism is a non-disease-causing change of a single base in the DNA. Therefore, when the MLPA method finds a deletion of a single exon, confirmation of the single exon deletion by another method is required.

If no mutation can be found with the MLPA test, the patient has probably a point mutation. With the *single condition amplification/internal primer* sequencing tech-

nique, *SCAIP*, developed in Dr. Flanigan's laboratory, it is possible to reliably find and characterize in detail all point mutations, including stop codons, small deletions and insertions, as well as splice-site mutations. In this two-step method, the complete base sequence of all exons of the dystrophin gene, as well as of all intron-exon border regions with the splice signals, and also of all promoters can be determined. All these separate gene regions are first amplified in one single so-called *polymerase chain reaction*, PCR, and then directly sequenced using standard automatic gene-sequencing methods to detect the point and other mutations.

The experience with the two test methods, MLPA and SCAIP, has shown that 93-95% of all mutations can be found using blood samples. However, the remaining 5 to 7% of all mutations cannot be found with them. If the patient has definite Duchenne or Becker symptoms, but no detectable mutation from blood samples, then a muscle biopsy becomes necessary so that the presence or absence of the dystrophin protein itself in the muscle fibers can be determined by one of the two protein detection methods, western blot or immunofluorescence. Western blot has an advantage in giving an indication of the amount and the size of the dystrophin protein, but immunofluorescence is widely available. Muscle tissue can also be used to extract messenger RNA, which can be used to find the rare mutations which cause incorporation of intronic fragments among the exons in the assembled DMD gene.

Because precise data on dystrophin-gene mutations of Duchenne and Becker patients and their exact clinical symptoms become increasingly important not only for the patients themselves but also for therapeutic research and the design of clinical trials, Dr. Flanigan's laboratory is collecting these data for a general diagnostic data bank in cooperation with clinical centers at six other universities in the United States. But also clinical centers and individual specialists for neuromuscular diseases from other parts of the United States and even from other countries are invited to send their patient data to the Utah center. More and more clinical trials will be performed in the future which will need participants with defined mutations and clinical symptoms. Based on the information of this data bank, the center may then recommend the inclusion of certain patients in these trials. Information on the inclusion of patient data for this bank, on the test possibilities and prices can be found on the Internet at [www.genome.utah.edu/dmd](http://www.genome.utah.edu/dmd).

## An interview with Stephen D. Wilton

Professor Wilton is Head of the Experimental Molecular Medicine Group at the Centre for Neuromuscular and Neurological Disorders of the University of Western Australia in Nedlands near Perth. On 16 July 2006, after the conference, *Steve Wilton* answered my questions, printed in italics, about exon skipping and the general state of research for a Duchenne therapy. The full name of the potential exon-skipping drugs, *antisense oligoribonucleotide*, is abbreviated as AON or oligo.

**Clinical trials with the exon-skipping technique are beginning.** *To begin, would you please explain to the*

*families with Duchenne boys, what the very promising results of exon-skipping research will mean to them who are waiting desperately for a cure of this terrible disease.*

I would not use the word cure, exon skipping would never cure Duchenne. At best we could reduce the severity, and in some cases, we may be able to reduce it a lot.

I would rather be cautiously optimistic and say that if we are going to make a difference with exon skipping, it would be a modest difference. And if it works better than we think, that will be great. It is very important, to keep the expectations very tempered.

*I am discussing exon skipping quite often with parents.*

*I explain it to them as well as I can, and always add that it has only been tested in animals, and that nobody can say at the moment whether it will work in boys as well. Then I explain that clinical tests with Duchenne boys are being done now which will show whether exon skipping will work in children or not.*

This is why at this particular meeting I was saying that our lab was able to induce skipping of every exon in the dystrophin chain except exon 1 and exon 79. All the exons from 2 to 78, we can skip. Many exons are easily removed, and a few are more stubborn but we are developing ways to skip the difficult exons, too.

Our clinical trials have to be done slowly, steadily, and step by step and, as soon as one step is taken, we are ready to take the next one. There are no great safety problems expected as just a single muscle is being treated and then later removed for analysis. There will be a greater risk when the trials progress to a whole-body treatment as much larger amounts of AONs will be administered and there is a chance of some unanticipated side effect.

But one does not know how long the dystrophin lasts after two or four weeks of the initial treatment. In the proposed British trial, one to four weeks after the treatment, the entire muscle will be removed and assessed first by molecular testing to see if dystrophin is present. This will be a proof of principle to show that exon skipping is going to work in humans.

At this time, I think, one should not experiment with different doses or times, because the real proof will be a whole-body treatment, a systemic treatment to demonstrate that exon skipping really works in human muscles. And that is going to be very, very difficult, because we do not know how much AONs we will need, and how often they will have to be administered.

For exon skipping, we will have to look at different compounds for different patients addressing different mutations. Each patient will respond differently. It is going to be very, very difficult research. But I may be overly pessimistic here.

*The Dutch researchers in Leiden are now trying to skip exons 51 and 46 and the British will also skip exon 51. And this will be extended to other exons. Will one have to go each time through the whole lengthy approval procedures?*

There will have to be approvals, because technically each new oligo is a new drug. But what we are hoping is that the oligos for the first trials are select examples, and that for the subsequent oligos the approval procedures could be shortened, if all oligos behave similarly and do not induce any side effects.

We, and I mean the British MDEX consortium, are working with modified oligos of a chemistry called *morpholinos*. This type of compound has already been systemically administered to humans as potential antibiotics. They have been shown to be safe, because they are not broken down in the body, they seem to be perfectly stable. Already extensive trials have been done with them, and thus they may not need such extensive clinical tests as, for instance, the other modified AONs, the Dutch researchers are using, the 2O-methyl-thioate-protected ones, which have not been administered to humans.

The morpholinos, on the other hand, have a completely different backbone and because they are so weird, there is

no way they can be integrated into the genome. So, what we are doing is really not a gene therapy, but rather a modification of gene expression.

If the first few morpholinos are shown to be safe as muscular dystrophy drugs, I would hope that the people in charge of drug regulation would relax the guidelines on what has to be done for new oligos. If we had to do extensive testing for every oligo, we might as well stop now, it would just be completely unsustainable and we would not be able to treat many of the different mutations causing Duchenne muscular dystrophy.

One of the most important aspects to consider is, that the oligos are not simple conventional drugs, but that we would be offering a personalized treatment, a customized molecular therapy for a boy with Duchenne.

Now, the company **AVI Biopharma** in Portland, Oregon is developing morpholino oligos as antiviral agents, which could potentially go to hundred of thousands of people. And therefore, with something going to the general population, you must have extensive safety testing to make sure that 0.1% of them do not have any unexpected adverse reactions. In contrast, our Duchenne oligos would be administered only to very specific cases of Duchenne dystrophy. So if there is an adverse event, and we hope that won't happen, it would concern only one or two boys, and since these patients will be very closely monitored during the treatment, any adverse effects should be seen quickly and appropriate steps immediately taken.

There could still be effects dependent on the different nucleotide sequences of the oligos. We have to be aware of that risk. But this would be a case of weighing the risks against the benefits. Even if there were some adverse side effects with these morpholinos in addressing Duchenne mutations, the benefits of restoring some dystrophin expression could outweigh the risks. Steroids have numerous side effects but these are currently accepted as the best treatment option.

At this meeting, *Dominic Wells* showed that when he was just injecting AONs directly into the muscles of mice, he was getting very good exon skipping at the RNA level after 14 weeks. So these morpholinos are inducing exon skipping for 14 weeks. And the protein is going to be more stable than the RNA. So after 14 weeks, you should get a lot of dystrophin, and that may possibly last for up to 26 weeks, that is 6 months! I never expected that this would last as long. It is working better than anticipated. And if there were an adverse reaction after a boy is given a morpholino, perhaps there are ways of controlling that. And then, the boy would perhaps have an uncomfortable week, and then it is going to be 6 months before the next treatment. Again, we have to work out whether a treatment twice a year would be adequate. We could compare this with chemotherapy and radiotherapy in cancer treatment. There are terrible side effects. And they are accepted there because there is nothing else.

**Two types of antisense-oligonucleotides.** *The Dutch researchers are trying their oligos, the 2O-methyl-phosphothioates, to skip first exon 51, and you and the British will test morpholinos. But you will work together, will you?*

The Dutch are looking at a variety of different exons, and the English have done a comparison and found that compounds which we developed, in particular the morpho-

linos, were working very well, and decided to use them for their clinical trial which is still in the planning stage. They will try to skip exon 51, too, to have essentially a parallel study to the Dutch trial. So, we will be able to compare different chemistries, different sequences, and compare the efficiency of these different treatments. That really could not be done if we and they were trying to skip different exons. So, if both types of AONs are being shown to be safe, work on both of them is going to be pursued. Hopefully, both will give similar results.

No one really knows what is going to happen when there is a long-term exposure to any of these different oligos. It is even possible that one treatment will need a combination of oligos. The idea is, that competition is healthy, and that we don't keep all the eggs in one basket. If both systems are promising, both have to be pursued.

And again, if something happens after three years of morpholino treatment, then we will fall back onto the 2O-methyls. But if we have just nothing than the morpholinos at that moment, we would be in trouble. But in addition to the morpholinos and the 2O-methyls, there are other types of AONs available with other chemistries we are working with.

**The first exon skipping experiments.** *Who actually had the first idea about exon skipping?*

I think it was developed simultaneously at several different places. I had been doing work on revertant fibers with dystrophin that appear spontaneously in Duchenne muscles, and was trying to work out what the mechanism was. And to me it was logical that it was some exon skipping mechanism. That was about in 1994, and we found some gene transcripts, some functioning mRNA for shortened dystrophin. And then at the end of 1996, at a meeting in Lake Tahoe, I met *Ryszard Kole* from the University of North Carolina. He was talking about suppressing the abnormal splicing in the  $\beta$ -globin gene as a therapy for thalassemia. As far as I know, he was the first person to modify the expression of a gene by modified splicing. Afterwards, Ryszard and I were talking quite some time, and then one of those moments happened where something hits you almost literally like a brick! It was just so obvious, that this was the way of going. If you could use AONs to suppress abnormal splicing, why not apply the same approach for normal splicing, too? Within a few weeks of getting back to Perth, I received some oligos from Ryszard. We had some cultures with muscle fibers going that were not very good, but we did some experiments anyway right away, and we got exon skipping in our very first experiment with one of Ryszard's oligos.

**When will there be an exon skipping therapy?** *The gene was found 20 years ago. Everybody was very excited then that next year, we would have a cure. What can one actually say to the parents who feel that the time for their boys is running out? Two years ago, Gertjan van Ommen of Leiden University told me in an interview, that it will take about 10 years until exon skipping will work for the boys. Two years are gone, so eight years are left. I have asked our families whether they really wish to know that, and their answer was: "Yes, we wish to know it, and we understand that estimates like this cannot be precise, and that this estimate does not mean that in January 2014 exactly*

*there will be something for our son." What is the situation now? How long do you think it would take?*

Yes, I know these are terrible things. This is the sixth year that I am coming to the Parent Project meetings. Unfortunately, I meet new people each year, and each year, some people are not here any more. But we have to proceed slowly and carefully and one step after the other to avoid mistakes that would make the time to wait even longer.

However, I am optimistic that, if the first clinical trials are done carefully and safely, then very quickly we plan to follow up with new trials. And the new trials would include new targets, that would skip other exons to address different mutations. So there will be no waiting for years to see what happens and analyzing the results. If we get a positive result in one trial, we will always start the next stage as soon as possible.

And one way how we might accelerate the work is, that instead of trying to skip only one exon at a time, we might target two exons or even more simultaneously, thus make multi-exon skipplings. Why not using a cocktail of oligos to skip several exons at once? We have actually done this in cell culture with a mixture of oligos to remove exons 50, 51, 52 and 53 simultaneously, and it works reasonably well. And another mix we have worked with, addressed exons 6, 7, and 8, which are the exons the dystrophic dog needs to have skipped. So we could try it on the dog before working with humans. Many different mutations could be addressed by one of these cocktails. And one advantage of using a cocktail is that you could do safety testing for three or more oligos at one time. And we could even combine different oligos. That would accelerate research considerably.

So, as it looks now, Gertjan's estimate is quite realistic, maybe the time will be shorter, five to six years from now on until we can treat the first boys with good results.

*When talking to families, I often hear them saying: "Why did Duchenne hit us? What is the reason?" "The reason are the mutations, and they occur at random, they cannot be predicted", I answer. But mutations are just tools of evolution. If there were no mutations, we would not be here, there would not be real life on earth, perhaps just slime. But evolution also made scientists - like you - who are trying to right this, to repair the gene, to solve that problem by finding a therapy.*

It is an interesting way of looking at it. It is true, without evolution, we wouldn't have even evolved. To look at it, everyone is different, everyone has probably slightly different dystrophin genes. In some cases there are single base changes in the protein coding exons, but these change one amino acid and are generally not significant, unless that was one very important amino acid. Every dystrophin gene is subtly different, and the genetic variation extends to other genes and the genes that control gene expression. We are a very complicated genetic package and that is likely to be the reason for the different clinical symptoms we find in different Duchenne patients.

**Manufacturing the oligos.** *Who actually makes the oligos? They are probably made automatically.*

I personally made the 2O-methyl oligos we used at the beginning with a machine we have in the lab. I pressed buttons to key in the AON sequences, I filled the reagents

in and kept them topped up, and I lost lots of sleep, when I had to watch the synthesis. The chemicals were very expensive and I hate wastage so the synthesizer was kept running around the clock. Now, there are many companies who make oligos but I prefer some control over the process. Once we optimized our 2O-methyls in cultured cells, we got in contact with AVI Biopharma in Oregon, the company that now makes our morpholinos even to clinical grade for the trials.

*Will these oligos be expensive when everything is ready for the boys?*

They will be expensive, but not as expensive as the production of viruses for gene replacement. Exon skipping will be many times cheaper. The cost of making these oligos is substantial, and we still need lots of morpholinos with different sequences. But if we can design oligos that work very efficiently in small quantities, which you can administer at a low dose and still are therapeutic, then these drugs might not be so expensive.

**Early preclinical diagnosis will be important.** *If exon skipping or another technique works, should it not be applied early before the muscles disappear?*

Early detection will be important. And possibly, if exon skipping was to work and shown to be safe, then, after an early diagnosis, you might start treating before any symptoms at all. And that could make a big difference. So I think an early diagnosis is a good idea and newborn screening for muscular dystrophy should be made available everywhere.

**Then there is a message of hope?** *At the end of this interview, would you please say something to the parents to keep hope after an important meeting like this one in Cincinnati?*

First let me give you a surprise: The progress with the morpholinos, that has been made in the last year, was astounding, we were getting results beyond our expecta-

tions. We are optimistic that they will work far, far better than we anticipated. At the beginning of our work, we found that they worked very poorly in cell culture. And when we started the work in mice with in-vivo injections, we were doing all these fancy tricks to get them into the muscles. It was working in principle but then we just tried a type of negative control, the morpholinos in saline, just in a 0.9 % common salt solution, and that worked beautifully, without any carrier to enhance delivery. The pure morpholinos in saline, the simplest possible way of administering it, worked very, very efficiently!

And then the people at AVI Biopharma attached some little peptide groups on them to enhance delivery even more, and these things work very, very nicely in the mouse. Now, we have to try this in humans. The people of AVI are developing new chemistries, making new modifications, and it is a wonderful collaboration with them.

But to finish, I want to say that this Parent-Project meeting has been very positive. There are so many different approaches of research for muscular dystrophy at the moment. There are pretty good trials on gene replacement, on read-through, there are two trials on exon skipping, there is the myostatin work, there are so many different things that are going on, and the steroids are being studied in great detail. There is reason for a lot of hope.

But it is never going fast enough. I wish we had a cure yesterday. But if we can make a difference soon, it will be by exon skipping. That will buy some time until something better or more permanent comes along. It is not a perfect treatment. But it is the best we can do at the moment with lots of oligos and with no completely new technology.

*Thank you very much, certainly also on behalf of the boys, their parents and their relatives, their doctors and the others who care for them, the Duchenne researchers and perhaps even influential people who could change things so that you and your colleagues get the funds and the opportunity to reach our goal soon, a therapy for Duchenne muscular dystrophy.*

### Concluding words by Pat Furlong

My two boys, Christopher and Patrick, were diagnosed more than 22 years ago. At that time the field was empty, there was no hope and no help. The dystrophin gene was elusive. In 1986, it seemed the world changed. The dystrophin gene was identified with great fanfare and it seemed treatment was on the horizon. Very soon after discovering the dystrophin gene, we recognized that the path was not as clear as initially believed. We had much to learn. In the last five years the world of Duchenne muscular dystrophy has changed dramatically and we, the parents and family members, have been instrumental in this change. The field is now ripe with possibilities and in these next few years,

we will see a number of promising clinical trials hopefully leading to the development of treatments. Duchenne will be removed from the list of 'lethal' conditions. I don't expect we will be magically removed from the world of Duchenne, rather I believe Duchenne will become a chronic condition that will require careful medical management. But it will be managed and these emerging treatments will preserve and protect muscle function. The Duchenne community and its progress will become a model for others with rare conditions and our sons will survive, thrive and reach their dreams

This report, written in Cadaqués, Spain, in August and September 2006, is also available in Spanish and German. All my earlier reports in English, German, and Spanish can be seen on the Internet at [www.duchenne-research.com](http://www.duchenne-research.com). Those who wish to receive my future reports by e-mail as soon as they are ready, should please send me their e-mail address.

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## Exon Skipping, an Example.

Here, the molecular details of skipping exon 46 are explained with which the Duchenne muscular dystrophy caused by the exon 45 deletion is changed to a Becker muscular dystrophy.

Part of the base sequence of exons 45 and 46 of the mRNA of the dystrophin gene is shown as well as the end of exon 44 and the beginning of exon 47. In exon 45, 50 triplets are not shown and 30 in exon 46. Below each triplet (codon), the abbreviated name of the amino acid is shown according to the genetic code. The triplets follow each other without spaces, the hyphens indicate here only the reading frame and the vertical lines the borders of the exons. The exon-skipping “therapeutic” oligoribonucleotide attaches itself to the underlined 19 bases in exon 46 of the pre-mRNA. The three bases of the hidden stop signal are also underlined. Exon 45 ends after the second base of the last triplet, which then is completed to AGG by the first base of exon 46 (-AGG-AG | G-CUA-).

<b>End Exon 44</b>		<b>Start Exon 45</b>		<b>End Exon 45</b>		<b>Start Exon 46</b>
-UGG-UAU-CUU-AAG		GAA-CUC-CAG-GAU---		AGA-AAA-AAG-AG		G-CUA-GAA-GAA-
trp tyr leu lys		glu leu gln asp		arg lys lys arg		leu glu glu
hidden stop code		antisense oligoribonucleotide				
		<b>GUC-GUU-GAU-UUU-UUU-UUC-G</b>				<b>End Exon 46</b>
--AAU- <u>GAA</u> -UUU---		AAA-GAG- <u>CAG-CAA-CUA-AAA-GAA-AAG-CUU-GAG-CAA-GUC-AAG</u>				
asn glu phe		lys glu gln gln leu lys glu lys leu glu gln val lys				
<b>Start Exon 47</b>						
UUA-CUG-GUG-GAA-GAG-UUG---						
leu leu val glu glu leu						

If only exon 45 is missing in the mRNA, the reading frame in exon 46 is shifted one nucleotide to the left, exon 46 then starts

instead of		G-CUA-GAA-GAA-C	with the sequence		GCU-AGA-AGA-ACA
		leu glu glu			ala arg arg thr

with the consequence that 16 incorrect amino acids are incorporated into the dystrophin until finally a premature stop signal UGA is reached which was hidden before (-AAU-GAA-UUU- is changed to -AAA-UGA-AUU-, the hidden UGA is underlined above). The protein synthesis is interrupted prematurely, the dystrophin remains incomplete, and *Duchenne muscular dystrophy* develops. After the deletion of exon 45, exon 44 is followed directly by exon 46:

<b>End Exon 44</b>		<b>Start Exon 46</b>
-UGG-UAU-CUU-AAG		GCU-AGA-AGA-ACA---AGA-UUU-AAA- <b>UGA</b> -AUU-UGU-UUU-AUG-
trp tyr leu lys		ala arg arg thr arg phe lys <b>STOP!</b>

If in addition to the missing exon 45, exon 46 is also removed, the reading frame is not disturbed, there is no premature stop signal, but 108 amino acids are missing in the central part of the dystrophin, which, however, is still partly functional. This changes the severe Duchenne muscular dystrophy in the much less severe Becker muscular dystrophy.

<b>End Exon 44</b>		<b>Start Exon 47</b>
---UAC-AAA-UGG-UAU-CUU-AAG		UUA-CUG-GUG-GAA-GAG-UUG---
tyr lys trp tyr leu lys		leu leu val glu glu leu