

PPMD Annual Connect Conference, Philadelphia, 12 - 14 July 2007

Working together to end Duchenne.

More than 400 people came to Philadelphia to attend the 2007 annual conference of the American Parent Project Muscular Dystrophy, PPMD. Over three days, about 60 presentations on therapeutic research, medical and social management, and legal affairs were on the program. I, *Günter Scheuerbrandt*, a biochemist from Germany, was asked by *Patricia Furlong*, president of PPMD, to write a report on this meeting for you, the Duchenne boys and young men and their families, who wish to know how the work of the researchers and other experts is progressing to find effective therapies for Duchenne dystrophy.

This report is my third one on PPMD meetings, the other two were on the PPMD meeting in Cincinnati in July 2006 and on the PPUK meeting in London in October 2006. You can see these earlier reports on my internet pages at www.duchenne-research.com and download them from there as pdf files.

The report contains the summaries of only scientific presentations because I am not a medical or social expert. It is not a scientific publication, and I have tried to write it in a way that will let you understand what is happening in the laboratories.

In the summaries, I am using the names of the presenters without their titles, most are professors and 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. 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 most have done so.

Introduction

At the beginning of the meeting, *Richard Finkel* of the Children's Hospital of Philadelphia, *Dominic Wells* of the Imperial College in London, and *Steve Wilton* of the University of Western Australia in Perth discussed in detail the general facts about Duchenne muscular dystrophy and the different research strategies. As my report on last year's meeting in Cincinnati started with a similar introduction, I have shortened those earlier paragraphs, updated them with new information, and repeat them here to help you to understand how genes make proteins, why dystrophin is so important, which research approaches are being actively followed, and how exon skipping works.

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 contains two of four different small molecules, the **bases**: *adenine, guanine, thymine, and cytosine* (A, G, T, C). For spatial reasons, the rungs can only contain two base combinations, the **base pairs** A-T and G-C. If e.g. -GGCTTAATCGT- is the sequence of these *bases* on one strand the sequence on the opposite strand must be

-CCGAATTAGCA- so that both sequences are *complementary* to each other:

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

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

ribonucleic acids, **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 muscle cells. The gene has seven or possibly eight different **promoters**, base sequences to which regulating proteins bind and thus activate the gene by initiating 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, at last count, 20,488 human genes, it fits into a cell nucleus of about 0.01 mm diameter only because the genetic material is extremely tightly packed. One molecule of the full-length dystrophin protein is much shorter than its gene, it is 125 nanometers (= 0.000125 mm) long, 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, at least about 30% of the normal number of the dystrophins has to appear again after the damaged gene cannot make them any more. The new ones don't have to have exactly the same form, they can be shorter, but they must be able to work properly. And that means billions and billions of new dystrophins have to come back in every gram of muscle, and a child has many kilograms of them!

The role of dystrophin. Dystrophin is needed for the

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

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

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

Boys with the slower *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, but also the smooth and heart muscles. Damage to the heart muscles produces *cardiomyopathy*, and the weakness of the smooth muscles has many consequences, among them the reduced ability of blood vessels to relax when blood flow increases leading to respiratory and other problems, and also the gastrointestinal tract is affected when the motility of the intestines is reduced. So the 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. Other mutations are inversions and changes in introns that alter normal splicing patterns.

As the three-letter codons of the messenger RNA is read in the ribosomes one after the other without interruption, this normal **reading frame** is not disturbed if

the mutation results in deletion or addition of entire codons of three bases each. In this case, the reading frame remains **in-frame** and the dystrophin can still be made but it is longer or shorter than normal. If this change affects only non-essential structures of the dystrophin, it can be partly functional and thus give rise to the less severe **Becker muscular dystrophy**.

If, however, the mutation shifted the reading frame by one or two base pairs, the reading frame becomes **out-of-frame**. Then, a 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 fulfill its normal function, it disappears and **Duchenne muscular dystrophy** develops.

Clinical course of Duchenne muscular dystrophy.

The first clinical signs appear at about two to three years of age causing difficulties in walking and especially in climbing stairs. Without early detection, even today the disease is generally diagnosed at about three

to five years of age. Because of increasing contractures at the foot, knee, and hip joints, the patients lose their walking ability at 10 to 12 years. Increasing spine deformities, scoliosis, and restrictions of movement make them soon dependent on permanent care. The involvement of the respiratory and heart functions lead to death by cardiac and circulatory insufficiency at an early adult age. Treatment with corticosteroids, physical therapy, orthopedic operations to avoid contractures and spine deformities as well as respiratory aids and other management measures can improve the quality of life and significantly prolong life expectancy.

Some of the medical and social care methods were discussed at this and the earlier Parent-Project meetings. I hope that there will be a possibility to write a report similar to this scientific one in the future by a team of specialists, because it will be needed by Duchenne boys everywhere to become aware and to take advantage of what has been achieved during the last years to reduce suffering, to extend their life and make it more meaningful.

The different strategies for a therapy of Duchenne muscular dystrophy.

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

The *first genetic approach*, **exon skipping**, 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. With this technique, the splicing of the exons of the pre-mRNA to the mRNA is specifically altered so that the disrupted, *out-of-frame* genetic 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 different groups of patients, can perform this change of genetic information: **antisense oligoribonucleotides, AONs**.

The *second 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 about one third as long as normal would fit in

them. This means that the new dystrophin will also have only about 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 slowly progressive Becker form with a relatively 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.

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 mostly 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.

Why do we need clinical trials?

How to get a drug to the patients. In her presentation with the title *Introduction to clinical trials*, **Kate Bushby** of the University of Newcastle upon Tyne explained how an idea of a scientist for a therapy, a new *hypothesis*, becomes a reality and leads to an effective drug for Duchenne muscular dystrophy. In this summary, I am repeating some of the most important general information given by Dr. Bushby at the 2006 PPUK meeting in London to-

gether with some new details mentioned at this meeting.

Clinical trials are the most important part of the development of a therapy process that takes many years. But Duchenne boys do not have many years to wait until a drug is available to them. But they and their families should understand that the scientists do appreciate their situation and are working as fast as they can with many collaborators and with the regulators to be certain that the

drugs they seek are effective and safe. But from the beginning, in the preclinical stage, they have to work carefully and one step after the other, before the clinical trials can be started. And these laboratory experiments can take many years. For instance, after the first ideas in 1993 about exon skipping as a possible method to combat a hereditary disease, it took 13 years until this technique is now being used in the first clinical trials with Duchenne boys.

The first task of the scientists, who start to develop a therapeutic drug is to gather experimental data by testing their new idea on a model of a disease like Duchenne dystrophy, i.e., on isolated muscle cells *in vitro* in a laboratory dish, and *in vivo* on dystrophic mdx mice and GRMD dogs. To determine whether their method would change the disease process, the pathology, they determine precisely the biochemical and biological consequences of their proposed treatment like the activity of the enzyme creatine kinase, the structure of the muscles, i.e. their histology, the presence and the properties of dystrophin and its messenger RNA. With experiments on animals, an improvement of the muscular function can be established and the toxicity checked to see whether a new substance has a positive effect and that it is not poisonous.

But even high-quality and reliable results of preclinical experiments do not prove that a new compound, a potential new drug, would show the same results when tested in children. Although the mdx mice have no dystrophin in their muscles, these small animals are not really handicapped, their disease is much milder than the human one. The dystrophy of the much larger golden retriever dog, GRMD, is more like the human disease. The dogs are really handicapped and have difficulty rising. But still, all the results of experiments obtained with these animals cannot necessarily be expected to be the same when the experiments are done on children. *A child is not a large mouse or a two-legged dog!* For this reason, clinical trials with Duchenne boys are necessary.

Usually, the trials have to go through three phases, and it is important to note that in phases I and II the participants in the trial may not experience any clinical benefit as these kinds of trials are designed mainly to answer safety and limited efficacy questions: (1) *Phase-I* to test for toxicity, (2) *phase-II* to test for dosage and safety and some effect of the treatment, and (3) *phase-III* continues to answer safety questions but is designed to confirm a clinically relevant positive effect and prove efficacy of the treatment, that is, to show definitively that the new treatment makes a real difference, a functional improvement in the quality of life.

The clinical trials to find a Duchenne therapy present a number of special problems: (1) This disease is rare, therefore the pharmaceutical industry is not always interested, but their involvement is necessary for the development of a drug. They also need a profit motive to attract sufficient capital, so the *orphan-disease* regulations as described on page 18 of this report are important. (2) Because Duchenne dystrophy is quite rare, patients with specific mutations in their dystrophin gene will be scarce, and this problem is made worse because many patients may still not have full molecular diagnosis applied, including point mutation testing where a deletion is not identified. So parents should insist that the exact mutation in the dystrophin gene of their son is determined as soon as possible.

(3) National and international registries, which will contain the full diagnostic data of as many patients as possible from all over the world, are now being established. Details of the program can be found on www.treat-nmd.eu. The families should be encouraged to find out about these registries and to have the data of their child registered.

Duchenne muscular dystrophy is a complex disorder and an effective treatment for the lifetime of the patient will probably have to act on the genetic machinery that makes dystrophin in healthy muscles. Such a *genetic drug* will probably be a completely new type of drug, able to work for a very long time, to treat all the muscles of a boy, even those of the lungs and the heart. Therefore, the demands for the safety and the efficacy of such a Duchenne drug are very severe.

The supervision and regulations imposed by different authorities are there to protect the patients from damage and also their doctors from legal consequences of a possibly dangerous treatment. They have to make certain that a trial is adequate to answer the question being asked. The regulations should also ensure consistency and accuracy of the data for the final regulatory approval. The extensive paperwork, the long delays, and the large cost of clinical trials make sure that everything is being done correctly in the interest of the Duchenne boys and their families.

There are more negative than positive clinical trials, so no patient should stop or neglect the best possible medical care that is already available. And only correctly designed and performed clinical trials will bring an effective therapy within a reasonable time. Mistakes must be avoided at all cost: they would set back the entire research efforts and prolong the time the boys have to wait for a decisive and positive change of their future life.

Results of clinical trials must be precise and meaningful. *Richard Finkel* in his second presentation explained that in order to reliably evaluate clinical trials with Duchenne patients and to compare their results with those of other trials on the same or similar drugs, the researchers have to agree on so-called *outcome measures*. Examples of medical, genetic and biological properties to be measured are the creatine kinase activities, the presence and structure of dystrophin, the muscle functions like strength, endurance, respiration, heart performance, and also some aspects of quality of life of the boy. The measurements should allow to determine whether the results are statistically significant with a p-value of at least 0.05, which means, the likelihood that a result occurred by chance is less than 5%.

The methods should be standardized and able to determine whether the potential drug investigated causes changes in the symptoms of the disease, which are clinically meaningful and specific for the disease, Duchenne or Becker dystrophy. They should also be safe and easy to perform, sufficiently sensitive to identify small changes, and they should be acceptable to the patients, his parents, and to the regulatory agencies. Thus, and above all, they have to show clearly whether a proposed treatment would be in the interest of the child, that is, if not providing a complete cure, then at least a therapy that would slow down the progression of the disease.

Biotech companies need capital. *Jeremy Gelber* of Morgan Stanley Investment Banking discussed the financial

side of drug development for a rare disease like Duchenne-dystrophy. The average out-of-pocket expenses for development of a drug until approval is about \$300 million, or capitalized more than \$800 million. This money has to come from investors who accept the risk of failure but expect a profit, if a drug is successfully marketed. Investment companies are able to raise millions of dollars, they know the often newly founded biotech companies, they know the pharmaceutical markets and they advise the investors, their customers, about the risks and possible benefits. These specialists are very aware of how important the positive outcome of the clinical trials is. Therefore the trials have to be designed and performed very carefully, because a failed trial has a large influence on the share price and the market value of a company. A negative trial can even destroy a company and thus cause the delay of the development of a drug, all the Duchenne families are waiting for.

"Please let our son be part of the first clinical trials, we are prepared to do everything and to go everywhere, because then he obviously would have a chance for a cure". Many e-mails, some from far-away countries, reach me with this desperate plea. The following paragraphs are an answer to this question.

Only very few children, less than 10, participate in the first three clinical phase-I trials on Duchenne boys in the United States, in the Netherlands and in Britain. They are coming from the neighborhood of the clinical centers because the children have to be clinically checked repeatedly and they must have a precisely known mutation in their dystrophin gene.

Only one single muscle is being treated locally in these first trials. Even if the results are positive, i.e., if sufficient

new dystrophin appears without serious side effects, and if this single muscle is functioning better afterwards, then, in spite of this positive change, the children *will not obtain any therapeutic benefit*. Their muscular dystrophy will neither be cured nor slowed down! With these first trials, one wishes only to prove that two new methods – exon skipping and mini-gene transfer – are really working in human muscles. One looks only for a *proof of principle*.

In general, only when this has been proven, the next step, a systemic application will be tried. The potential drugs – antisense oligoribonucleotides, AONs, or adeno-associated viruses charged with mini genes – will be injected into the blood circulation so that they can reach all muscles. The first systemic trial will probably start in the Netherlands in 2008 and will be performed also with few children from the neighborhood of the clinical centers.

For these reasons, it does not make sense for the parents to travel with their child to the trial centers from far away or even from other continents and to live near them for many months. This would be much too expensive and would not cure the child. The best the families can do to get access to a therapy as soon as it is ready and approved is to be a member of one of the active muscular dystrophy associations, to have a gene analysis made for the child and to register his exact mutation and all other clinical information in the Duchenne data banks which are now being established. Then the researchers could contact families whose sons have a *needed* mutation because more and more clinical trials will be performed in the next years, also with boys who have a rare and unusual mutation. And the families should read up-to-date research reports so that they know what is going on and when and where a really effective drug will be available.

Exon skipping and gene transfer.

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

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

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

Becker muscular dystrophy.

For the first exon skipping trials, two kinds of chemically protected AONs are used. They have to be protected because then they are not or only slowly destroyed in the muscle cells by nucleic acid destroying enzymes. The two types of AONs are the *2'O-methyl-phosphorothioates*, also called *2'O-methyls* and the *morpholinos*. Because of their unusual structure, the morpholinos are not really nucleotides, so the abbreviation AON is not quite correct for them. But for practical reasons, I am using AON for both types in this report as is being done also in many scientific papers.

Exon skipping trial in the Netherlands. *Judith van Deutekom*, now head of research of Prosensa B.V., a biotechnology company in Leiden in the Netherlands, was unable to be present at the meeting in Philadelphia, therefore, *Elizabeth Vroom*, president of the international United Parent Project Muscular Dystrophy, UPPMD, reported on the *first in-human trial with the exon skipping technique* which was completed at the end of 2006.

The aim of this trial was to prove that exon skipping is feasible in Duchenne patients. It was a *local study* on a small area of a single muscle, the *tibialis anterior* muscle of the shin, which was being treated with an *2'O-methyl AON* against exon 51. The trial was designed to provide a

proof of principle only and not a therapeutic benefit to the treated boys.

The Dutch researchers selected the 2'O-methyl version of the anti-51 AON, because they have extensive experience with this type of chemically stabilized AONs, not only by successfully treating muscle fibers in cell cultures but also by local and systemic injection into individual muscles and the blood circulation of living animals. Exon 51 was selected for the first skipping target because successful skipping of this single exon would allow restoration of the protein reading frame for up to 25% of all Duchenne boys with deletions.

Because exon skipping is a new medical procedure, intensive clinical and molecular genetic tests were performed on each boy before the start of the trial. As the Dutch regulatory agency did not allow a muscle biopsy to be performed before the trial, a skin biopsy was taken from which cell cultures were prepared. In these laboratory procedures, the particular deletion, previously determined on the DNA level, was confirmed in the mRNA, and the base sequences of the border regions before and after the deleted exons were determined also. In addition, the entire dystrophin gene was screened to make sure that there were no unexpected irregularities. Although it was already known that the skipping of exon 51 works well in living animals and in cell cultures from Duchenne patients, the skipping procedure was repeated on the muscle cell culture from the skin biopsies of each boy, in order to exclude any risk that it would not work in the living muscle during the trial. For further safety reasons, the boys were treated sequentially, one after the other, meaning that only after the results for the first boy were positive and did not show any side effects, the second boy was treated, and so on.

The results of the trial, while very encouraging, cannot be summarized here because at the time of writing this report at the end of November 2007, they were not yet published. After their publication, they will be part of my next research report available in the first quarter of 2008.

The Dutch researchers are now preparing the next clinical trial during which they will aim at full-body, *systemic*, administration of the exon 51 2'O-methyl AON so that the potential drug can reach all muscles including those of the lung and the heart. These studies will be short-term, one month, and long-term, six months, and will be done with different amounts of AON to determine the most effective dosage which possibly could already slow down the boys' Duchenne symptoms significantly. How much the muscle function will be improved will also depend on the actual resulting functionality of the Becker-like proteins obtained after skipping.

Also, more systemic studies with mice will be performed to investigate the pharmacodynamics of the AONs to find out what exactly happens with them inside the muscle fibers. In some of these animal studies, the AONs will be injected subcutaneously, under the skin, because this will later be the most practical type of application if repeated injections are needed. In similar studies, it has been shown that the systemic application works well, that even the heart muscles can be treated, and that there are no serious adverse effects on the liver enzymes or other blood values.

The full development of exon 51 skipping is just the start of Prosen's research program to find an effective treatment for Duchenne muscular dystrophy. The devel-

opment of other AONs for other deletions will follow soon after. In addition to the 2'O-methyl AON for skipping exon 51, the company has already designed and produced in sufficiently large quantities other AONs for skipping the exons 43, 44, 45, 46, 50, 52 and 53. These AONs together would allow the treatment of over 65% of all patients with deletions.

In the future, it will also be possible to use this technique to restore the reading frame for some duplications or when more than one exon has to be skipped. E.g., the multiexon skipping of the 11 exons 45 to 55 would produce a Becker dystrophin in up to 63% of Duchenne boys with deletions as described by *C. Beroud et al.* in *Human Mutation* 28:196-202 (2007). The present state of treating duplications was described by *A. Aartsma-Rus et al.* in *BMC Medical Genetics* 8:43-47 (2007), and a review on exon skipping was published by *A. Aartsma-Rus and G-J. B. van Ommen* in *RNA* 13:1-16 (2007).

Exon-skipping clinical trial in the United Kingdom. At the meeting in Philadelphia, the clinical exon-skipping phase-I trial was not discussed which, after some delay, is about to start in the United Kingdom at the beginning of 2008. But because this trial is so important, I am repeating some of my summary from the 2006 PPUK meeting in London with more recent information added:

The *MDEX Consortium* was established in January 2005 in the UK with the aim to develop the exon skipping technique further and to perform clinical studies. The members of the consortium are *Francesco Muntoni, Kate Bushby, Volker Straub, Dominic Wells, Jenny Morgan, George Dickson, Ian Graham, Matthew Wood, Steve Wilton, and Jenny Versnel*. The UK Department of Health, the Medical Research Council, the Parent Project UK (now Action Duchenne), and the British muscular dystrophy association, Muscular Dystrophy Campaign, are also involved.

Francesco Muntoni of the Imperial College as chairman of the MDEX Consortium, said at the 2006 London meeting that in this trial, *exon 51* is attempted to be skipped, because about 20% of Duchenne boys, those with the deletion of exons 45-50, 47-50, 48-50, 49-50, 50, 52, 52-63 could be treated by skipping just this one exon 51. Six different antisense oligos, AONs, were tested in normal human muscle cultures, in muscle cultures from Duchenne boys, in entire muscle preparations (with *Steve Wilton*) and in the humanized dystrophic mice which contain muscle from Duchenne patients (with *Judith van Deutekom*). The best results were obtained with the *morpholino* AON *H51A* developed in *Steve Wilton's* laboratory. *Dominic Wells* could show in experiments with *mdx* mice, that this morpholino AON was sufficiently stable for a long-term treatment of Duchenne boys. This result was obtained by the cooperation of four laboratories, two British, one Dutch, and one Australian, it was published in September 2007 in *Human Gene Therapy*, 18:798-810. As mentioned before, morpholinos have a structure that is chemically different but have a similar shape as the 2'O-methyls, thus they are not really nucleotides, but in order not to make things too complicated, I abbreviate them also as AONs.

The morpholino AON to be used by the British, is 30 units long, it includes the entire sequence of the 2'O-methyl AON used by the Dutch, which has only 20 units.

Both antisense components attach themselves exactly to their complementary sequence of the exonic splicing enhancer, ESE, *inside* exon 51 of the dystrophin pre-mRNA and nowhere else. This blockade of the ESE inhibits the binding of SR proteins which are essential components of the RNA-protein splicing complex. The consequence is the exclusion of the targeted exon from the mRNA, i.e. the exon is skipped. Exon ESE sequences in different genes have some structure similarities, but they are sufficiently different, and this is the reason for the specificity of exon skipping: only the targeted exon is skipped in the targeted gene and no other exon in the same gene or in any other of the more than 20,000 human genes because it is statistically improbable that there is another completely complementary sequence of the length of the two antisense structures in the entire human genome. Therefore, it is unlikely that this genetic technique will cause any genetic side effects.

In the British trial, three groups of two Duchenne boys each, 12 to 18 years old, will participate. Three different dosages: 0.09, 0.297, and 0.9 mg morpholino AON in 0.9 ml solution will be used for each group, delivered into a volume of one cubic centimeter of muscle tissue with nine injections directly into one of the two *extensor digitorum brevis* muscles on the outside of the foot that is needed only to wriggle the toes. 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 30 days after the injections to assess the results of the treatment.

Like the Dutch trial, the British trial also will only provide the *proof of principle* 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 at least with some of the different dosages, shortened dystrophin will appear in more than 10% of the muscle fibers. The participating boys will not get any therapeutic benefit. But all the results of this trial will be needed later for a systemic application of the potential Duchenne drugs into the blood circulation so that all muscles can be reached.

After a delay of several months, the last of the necessary permissions by the UK regulation authorities has been given in October 2007, so that the first boys will receive their AON injections at the beginning of 2008 after completion of all pre-clinical preparations.

Janet Rose Christensen is Vice President for Regulatory Affairs and Quality of the company AVI BioPharma Inc. in Portland, Oregon in the US. She explained the role of her company in the exon skipping trial with Duchenne patients in the United Kingdom. For many years, this pharmaceutical company has developed the morpholino antisense oligos, *morpholinos* for short, as genetic drugs against many cardiovascular, viral, and liver diseases. Eleven clinical trials with compounds of this kind have already been performed with over 300 participants. Thus the company has a large chemical and clinical expertise with these antisense drugs. Sequences targeting the dystrophin gene to induce exon skipping have been identified, in cooperation with *Steve Wilton* in Perth in Australia. With the MDEX consortium, they will be actively involved in the phase-I clinical trial now about to be started in the UK.

The morpholino, called AVI-4658 by the company, which will be used in this trial for skipping exon 51, is a chain consisting of 30 subunits. Research studies have shown it to effectively induce exon skipping of exon 51. It attaches itself very specifically at the selected complementary target sequence in the center of exon 51 using Watson-Crick base pairing. This morpholino consists of 1,144 carbon, hydrogen, nitrogen, oxygen, and phosphorus atoms. It is synthesized in AVI's clinical manufacturing area by trained people according to "*good manufacturing procedures*", GMP, for the production of drugs.

Before the AVI-4658 can be used in the British trial, it had to be approved by the MHRA (Medicine's Health Regulatory Authority) in the United Kingdom. *Janet Christensen* then described the complicated approval procedures in the United States: Because Duchenne dystrophy is considered a "severely debilitating and life-threatening disease", it is expected that the so-called "FDA regulations 21 CFR 312 Part E" will be applied which say in part "...it is appropriate to exercise the broadest flexibility in applying the statutory standards, while preserving appropriate guarantees for safety and effectiveness. These procedures reflect the recognition that physicians and patients are generally willing to accept greater risks or side effects from products that treat life-threatening and severely-debilitating illnesses, than they would accept from products that treat less serious illnesses."

Janet Christensen finished her presentation by saying: "These regulations empower the FDA to look a bit differently at these applications for a Duchenne therapy than they would look at another headache drug, and it is now important to get the first clinical trial with this morpholino underway and successfully completed. We hope that this will open the way for a much faster approval process for the many following which will be needed for treating all the boys whose disease can be treated by exon skipping."

First steps towards multi-exon skipping. *Terence Partridge* of the Children's National Medical Center in Washington discussed some open problems with exon skipping and then his and his colleagues' new research work to skip two exons in dystrophic dogs to open the way to multi-exon skipping which would bring this new technique also to the of Duchenne boys, who need more than one exon skipped to restore their shifted dystrophin reading frame.

Exon skipping in mdx mice works well with the 2'-*O*-methyl and *morpholino* AONs against exon 23 injected repeatedly into their blood circulation where they produce new dystrophin in some muscles of up to 50% of the normal level. But the morpholinos do not enter the heart muscles and the reason for this failure it is not known. *Dominic Wells* of the Imperial College in London reported at the 2006 PPUK conference in London on first experiments which showed that morpholinos enter the heart under ultrasound treatment. Dr. Partridge and his colleagues are also working on this problem.

Although mdx mice do not have any dystrophin in their muscles, their dystrophy symptoms are very mild, therefore, they are not an ideal animal model of the human Duchenne muscular dystrophy. And because they live only about two years, long-term experiments lasting several years cannot be performed with them. However, dogs live much longer than mice, and the dystrophic GRMD golden

retriever dogs are physically handicapped, thus experiments with these dogs would give results that would more likely be similar to what one would later see in clinical studies with Duchenne patients.

Experiments with GRMD dogs have another advantage: The two clinical trials in the Netherlands and the UK for skipping exon 51 in Duchenne boys have shown and will further show that exon skipping really works in patients. In these trials, only a single exon is being skipped. But about 50% of all Duchenne dystrophies caused by deletions, duplications, and point mutations mentioned in the Leiden Duchenne database will need the skipping of two or more exons to restore the reading frame. And some multi-exon skipplings would be a therapy for rather large groups of patients. For example, the skipping of the 11 exons 45 to 55 would produce a Becker dystrophy with very mild symptoms in up to 63% of Duchenne boys with deletions. And because the dystrophic dogs need a double-exon skipping, experiments with them would open the way for a treatment of those Duchenne boys with the more difficult mutations.

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

Different doses of a cocktail of three morpholino AONs, two different ones against exon 6 and one against exon 8, were locally injected into the tibialis-anterior muscle of young adult GRMD dogs. Two weeks after the injection, biopsies were performed, which showed that new dystrophin had appeared in all fibers of the muscle around the injection site and that the muscle structure looked almost normal.

Dr. Partridge and his co-workers have recently collaborated with *Shin'ichi Takeda* in the General Animal Research Facility in Tokyo, where they were able to perform systemic injections into the blood circulation of the dogs with very promising results. As the details of these experiments are not yet published, they cannot be summarized here.

Exon skipping with morpholino AONs have a number of advantages: (1) They lead to a body-wide delivery to all muscles, but still with the exception of the heart; (2) they are highly effective; (3) they do not seem to be toxic; (4) they are not metabolized, degraded, in the body but excreted with the urine; (5) they only have a short-time effect, so repeated treatments will be necessary, but this would allow to terminate the treatment if a better therapy becomes available; and (6), they do not create immune problems because they do not contain foreign proteins and the new dystrophin protein is only produced inside the muscles.

So morpholino 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. Most of the work in the past years has been done with the morpholinos and the 2'O-methyl AONs. A future and final therapy might very well need a mixture of both types of AONs to optimize the skipping effect and to minimize toxicity. And still unknown problems could appear which one would perhaps have to solve with other AON cocktails or even with other kinds of AONs.

But when will there be an exon-skipping drug for Duchenne boys? I asked *Gertjan van Ommen* in Leiden this question in an interview in January 2004. His rather long answer, here shortened to 7 words, was: "they will be ready in 10 years". The entire interview is in my 2004-1 Monaco report at www.duchenne-research.com. The ten years were not meant as a scientifically precise time interval. It was just an estimate by an outstanding scientist working on this new technique, who knew quite well what slow research steps were still necessary and that there are many things that could go wrong. Now, at the end of 2007, you could say that a little bit more than 6 years are left of the original 10, if you take these 10 years at their face value. But you are not allowed to do that! The number of the remaining years could be 8, 9, and even 10 again, or, if all goes well, as few as 4 or 3.

And I was told by some, that one should not give any time estimate, because you, the parents, would take this information literally and start to count backwards the months and days. On the other hand, I know that those, who have an idea of how the real world of life, and the world of science in particular, works, will understand this situation and appreciate the following statement, that I received from *Francesco Muntoni*, who wrote it after having discussed it with his colleague van Ommen:

"I entirely agree that it is important to provide realistic information; and it is realistic to expect that during the next 6 years the scientific community, after having skipped exon 51, will have completed more systemic trials of AONs to skip several additional exons. It is important to realize that an experimental path towards a therapy never stops. For instance, there are new antibiotics being produced all the time. I do not think that we will have to wait 6 years to see the first potential benefit; but equally I am pretty sure that the type of the AONs and their mode of delivery we are using now will be considered obsolete by then. It is therefore likely that during the next 6 years, we will see the development of an effective and reliable method for the repeated administration of several AONs, and hopefully this will lead to a significant and positive clinical response. At the same time it is also very likely that we, the scientific community, will be studying still better chemical structures and delivery methods, and we would not be surprised if in another 6 years, additional clinical studies will be launched to test new and improved AONs.

The point I really want to make is, that this is a broad path, not a single goal, and provided things are moving forward, this will be a very eventful path in the right direction. Sometimes things happen more rapidly than we expect, and these bursts of genius from someone that causes them, are always very welcome but totally unpredictable. We expect to learn much from the early AON trials, and what we learn will positively impact delivery, dissemination and efficiency, and significantly decrease the waiting time for so many who watch and hope.

Transfer of the dystrophin gene, first clinical trial with a virus vector. The following description is based on the summaries in my reports of the Cincinnati and London meetings about the presentations of *Scott McPhee* of the company *Asklepios Biopharmaceuticals* and *Xiao Xiao* of the University of North Carolina, both in Chapel Hill NC. The earlier information is updated

with the new data given at the meeting in Philadelphia by **Christopher Shilling** of the Center for Muscle Gene Therapy at the Children's Research Institute in Columbus/Ohio where the trial takes place.

The transfer of a modified dystrophin gene with a *viral vector*, a gene transporter, into the muscle cells is one of the strategies for a therapy of Duchenne muscular dystrophy. The use of adeno-associated viruses, AAV, as vectors has some important advantages. They are highly effective in muscle and heart and can probably be used to treat all Duchenne patients without regard of their mutations. Pre-clinical proof-of-principle studies in dystrophic mice and dogs have aided the development of a mini-dystrophin gene vector for the first clinical trial of this gene therapy method. The vector used is a AAV of serotype 2.5, a so-called *Biothropin*TM biological nano particle developed by the company Asklepius. These viruses cannot be multiplied by the cells they infect, because most of their genes were removed. This modification made room for the coding sequences of a therapeutic gene to be transferred.

However, the AAV vectors are rather small, they can only accept foreign genetic material that is not longer than about 5,000 base pairs. For this reason, the cDNA of the normal dystrophin, the combined 79 exons without the introns, which is about 14,000 base pairs long, had to be shortened considerably to fit into this small vector. A transfer of such a *mini-gene cDNA* would thus not cure Duchenne muscular dystrophy but may instead transform it into a much slower progressing Becker form.

Therefore, the vectors used in the first trial are carrying the mini-dystrophin gene construction delta-3990 with parts of exon 17 and all exons 18 to 59 and 70 to 79 deleted. That means the expected Becker dystrophin will be about one third as long as the normal protein. In 1990, a 61-year old Becker patient was diagnosed who was still able to walk and who had this kind of shortened dystrophin in his muscles.

This phase-Ia gene-therapy clinical trial is now 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 was approved by the Federal Drug Agency FDA following safety and toxicology testing of the AAV mini-dystrophin vector in laboratory animals. It has started on 28 March 2006, when the first boy received the first injections of Biothropin 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 scientific investigators know until the end of the entire trial into which biceps the vectors were injected.

No therapeutic benefit is expected for the boys at this first trial whose main objective is to provide the *proof of principle* that this type of gene therapy does not only work in a skeletal muscle of mice and dogs but in a human muscle as well. The other aims were to collect safety data, to determine the dose required to achieve the production of the mini-dystrophin in muscle; and to see whether there are any immunological responses to the mini-dystrophin or to the vector material.

Six Duchenne boys who are at least five years old and whose mutations of the dystrophin gene are precisely known, were participating. All six have now received their injections. Two different doses were used for each

group of three patients. Samples of muscle tissue from the injection site were obtained by biopsies from four boys at four weeks and from two boys at twelve weeks after the injections.

No gene therapy-related adverse events were observed, suggesting that the procedure is well tolerated. Due to the nature of double-blinded design, the muscle biopsy samples were kept frozen until the end of the entire trial when they were checked for the presence of the new but shortened dystrophin. Results from these analyses will be available at the end of 2007.

The next, so-called bridging or phase-Ib studies are now being prepared with dogs and monkeys. Encouraging gene transfer and expression results have already been obtained. The bridging study with Duchenne boys will hopefully be performed in 2008 and 2009. In this trial, a whole limb will get infusions of the vectors through the temporarily blocked blood circulation. This 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.

Gene transfer with pericytes, adult muscle stem cells.

Giulio Cossu of the Stem Cell Institute at the Hospital San Raffaele in Milan described his and his colleagues very promising work towards a stem cell therapy for Duchenne dystrophy.

For an effective stem cell therapy of Duchenne dystrophy, a safe and ethically acceptable source of a large amount of adult muscle stem cells is needed, that would give rise predominantly to muscle cells and not to undesired cells such as 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 vessel and muscle cell walls, and stay inside the fibers without creating any local trouble. These conditions seem to be met by *mesoangioblasts*, which are adult stem cells located on the outside of small blood vessels within muscle tissue from where they can be isolated.

For their first experiments in animals, the Italian researchers used a mouse with a type of limb-girdle muscular dystrophy, which had one of the proteins of the dystrophin-protein complex missing. After injections of mesoangioblasts from normal mice, these "healthy" stem cells were able to cause the re-appearance of more than 80% of the normal amount of the missing protein, *alpha-sarcoglycan*, in all the skeletal muscles of the dystrophic mice.

For a possible Duchenne therapy by this new technique, the intact gene for dystrophin has to be transferred from the muscle tissue of a healthy donor into mesoangioblasts of the patients in a laboratory procedure with a viral vector. Then some transduced cells which contain the normal dystrophin gene should be multiplied and finally re-injected into the blood circulation of the patient. Such an autologous treatment would avoid immunological problems. Alternatively, mesoangioblasts from a healthy relative with normal dystrophin genes may be used. But this kind of heterologous treatment would need long-term immune suppression.

The mesoangioblasts used in these experiments were isolated from the blood vessel walls of mice and dogs. But

to perform the next step towards the development of a therapeutic method for Duchenne patients, such stem cells must be derived from a human source to be used in experiments with animals. Dr. Cossu and his colleagues have indeed done this during the last months and published their new results in February 2007, *Nature Cell Biology* 2007: 9, 255-267.

Therefore, the researchers looked for similar stem cells in the walls of the small blood vessels of human muscle tissue obtained from diagnostic biopsies. They found such stem cells there, but their properties were somewhat different from those of the mesoangioblasts. Therefore, these cells were called "pericyte-derived cells", or *pericytes* for short. Their properties were exactly those which stem cells should have when they were to be used for a Duchenne therapy, namely: (1) They are easy to isolate from human biological material like muscle tissue; (2) they can be multiplied substantially in the laboratory to amounts necessary for a systemic treatment of children; (3) it is possible to transfer into them "healthy" dystrophin-gene sequences with viral vectors; (4) they are able to migrate from the blood circulation into the muscles; and (5) they develop to functional muscle cells inside the living muscle tissue.

The most decisive results were obtained in experiments with mdx mice which, in addition of not having any dystrophin, also had their immune system inactivated by genetic manipulation. One month after three systemic injections of normal, that is non-dystrophic, pericytes into the leg arteries of five of these mice, 200 to 450 new dystrophin-containing muscle fibers were found per standard cross sections investigated. When human pericytes from Duchenne patients with mutated dystrophin genes were first treated with virus vectors containing mini-dystrophin genes, were injected similarly into these mice, 190 to 320 new muscle fibers with mini dystrophin were found in the investigated muscles. The function of the muscles in the treated mice were found to be significantly improved.

Therefore, as the next step towards a human application, Dr. Cossu's team isolated mesoangioblasts from normal and dystrophic dogs, multiplied them in culture and injected them into an artery of the dystrophic dogs' hindlegs. The dog cells were not characterized in great detail and so it was not known whether they really corresponded to pericytes as in humans. For this reason they were again called mesoangioblasts.

Four dogs were treated with five monthly systemic injections of autologous cells isolated from each treated dog, into which the gene, the cDNA, of a human *micro-dystrophin* was transferred with a viral vector in the laboratory. In this case, no immune suppression was necessary. But,

against expectation, this treatment was not successful. The reason for the failure may have been that the short length of the dystrophin produced after the transfer of the micro-dystrophin gene was insufficient to preserve the muscle function in a large animal like the dog.

In a heterologous treatment, six other dogs were similarly injected with donor cells from healthy dogs but with immunosuppression by cyclosporin. In this case, an extensive expression of normal dystrophin and an amelioration of the muscular function was obtained. In one animal, the cells were released from a catheter into the aorta. This allowed more widespread dissemination of the cells. The results of the stem-cell infusions were dramatic: this last animal displayed a marked improvement in its dystrophy and was walking well five months after the final injection; the other animals recovered to a lesser degree.

The researchers plan to repeat the experiments with the autologous cells after transfer of a mini-dystrophin genes instead of the shorter micro-genes, and possibly also to use the genetic exon-skipping method developed by *Luis García* in France.

Dr. Cossu finished his presentation with the statement: "We now propose a clinical trial with Duchenne patients. But first we have to perform long-term studies with the dogs and we have to do toxicology tests and to prepare the human cells under clinical grade conditions. This work has already started and will take another year. In the trial, three patients will get injections locally into one muscle to see whether these human stem cells really produce human dystrophin in a human muscle. Thereafter, three other patients will receive systemic injections of donor cells together with drugs against immune rejection of the foreign cells.

The first and most important outcome measure will be safety over a long time. We already know that nothing happened to the mice and dogs, but we do not know what will happen to the boys when we really treat them in a clinical trial. The second outcome measure will be to see whether muscle force is preserved or even increased. We have developed a new method to measure the force in single muscle fibers. And, obviously, we will follow all TREAT-NMD recommendations.

This stem cell treatment is still in its infancy. We do not know how effective it will be, and what will be the risks. After all, we will be injecting a large number of cells into the boys, and there might be serious problems. The procedure is complicated, it will take some years, and it will cost much. But it has a great potential, and we are excited about its possibilities. We will try it in children because it may lead to a real cure."

Pharmacological Approaches.

Upregulation of utrophin to replace dystrophin. There are four great challenges that have to be met by anybody trying to find a therapy for Duchenne dystrophy: (1) The function of the huge protein dystrophin which is missing in Duchenne boys, has to be restored; (2) to make a difference, at least 20% of the normal level of dystrophin has to reappear again, or if not this protein, then another one like *utrophin* which can replace it; (3) this must happen in all skeletal muscles, and in those of the heart and lungs, also;

and (4), any immune reaction against a new protein has to be avoided.

When *Kay Davies*, now at the University of Oxford, tried to find the Duchenne gene more than 20 years ago, she found instead the protein, *utrophin*, and its gene. 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. Like dystrophin, it connects the F-actin structure in

the cells with a protein complex in the membranes similar to the dystrophin-associated complex. Utrophin is present in many body tissues, also in human muscle, but there it is concentrated in regions where the motor nerves contact the muscle membranes at the *neuromuscular junctions*.

In Duchenne patients, utrophin starts to spread from the nerve-muscle junctions to the muscle membranes. The more utrophin a patient has, the later he must use a wheelchair. That means that a larger amount of utrophin by *up-regulation* of its gene would make Duchenne dystrophy more benign. During fetal development at 12 weeks, the muscles contain both, utrophin and dystrophin, then utrophin slowly disappears from the cell membranes, until at birth only dystrophin alone remains on the membranes. Thus utrophin is a fetal form of dystrophin. This means, that reactivation of the developmental program for utrophin would lead to a treatment for Duchenne dystrophy.

Mdx mice whose utrophin gene was *knocked out* experimentally, which have neither dystrophin nor utrophin in their muscles, have Duchenne-like symptoms and die early in contrast to "normal" mdx mice whose muscles show less severe damage in spite of the absence of dystrophin.

In other experiments with transgenic mdx mice which had utrophin mini-genes in their germ line, introduced by a technique that cannot be used in humans, it was shown, that utrophin, if it is present in larger amounts, can replace dystrophin. By increasing the amount of utrophin by a factor of three to four, the development of the rather light dystrophic symptoms of the mdx mice could be prevented.

Thus, for a possible Duchenne therapy, one should try to increase the low amount of utrophin by *up-regulation* of the activity of its gene. The gene has two different start sites, promoter sequences, to which signaling compounds bind to initiate the synthesis of the protein. One promoter induces the production of the predominant one of two similar forms of utrophin, the *A-utrophin*, which is then exclusively located in rather small amounts at the neuromuscular junctions of all muscle cells. The researchers then started to interfere with this signaling process, so that more of the A-form of the utrophin is made and directed to the muscle cell membranes where it would possibly occupy the sites vacated by dystrophin in Duchenne boys. However, it was soon realized that finding a substance that could upregulate utrophin and then developing it to a potential drug for an effective Duchenne therapy dystrophy was a difficult and expensive task that could not be performed in a university laboratory. So Dr. *Davies* co-founded the company *Summit plc*, formerly VASTox plc, located in Abingdon near Oxford.

At this company, by the end of 2007, over 30,000 chemical compounds will have been screened for their ability to upregulate the activity of the utrophin gene in tissue cultures from mdx mice. The light-producing enzyme luciferase from fireflies is used in a newly developed reagent system to test for the presence of utrophin. Up to July 2007, 31 promising substances were found which could increase luciferase several fold. They are now being optimized and tested in living mdx mice with the aim to increase the efficiency further and to assure that utrophin is sufficiently upregulated in all muscles of the animals. Additional screening tests with dystrophic zebrafish are ongoing which will possibly identify other pharmacological drugs for treating Duchenne dystrophy.

Two of the earlier active compounds, VOX A and VOX B, have already been tested systemically in mice by intraperitoneal injection, into the abdomen. After 12 weeks of weekly systemic injections, utrophin in the tested skeletal muscles of the mice were upregulated with evidence of muscle benefit. After further optimization of the compounds a clinical development candidate SMT C1100 showed reduced muscle degeneration, fibrosis, fat deposition, and chronic inflammation, thus the animals had significantly recovered their muscle function. After daily injections for 28 days, no side effects appeared, so the compound seems to be safe. If ongoing preclinical toxicology and manufacture is successful the first-in-man safety trials with healthy volunteers could take place in the middle of 2008 and with Duchenne patients could probably begin at the beginning of 2009.

Biglycan treatment upregulates utrophin. At the 2006 meeting in Cincinnati, *Justin Fallon* of Brown University in Providence, RI, described the work of his laboratory on the protein *biglycan*, now, at Philadelphia, he presented new results showing that biglycan can dramatically upregulate utrophin.

The protein biglycan is present during development at the outside of the skeletal and heart muscles and connects with its two ends the proteins alpha- and gamma-sarcoglycan, which are two components of the dystrophin-protein complex in the muscle cell membranes. Biglycan is important for the regulation of many signaling and structural proteins of the membranes. Experiments with non-dystrophic mice whose gene for biglycan had been deactivated, showed that in the absence of biglycan many proteins of the dystrophin complex had disappeared. Treating these mice with local and systemic injections of recombinant, artificially made, human biglycan led to the re-appearance of the protein beta-syntrophin, which was an indication that the dystrophin complex was restored.

Human biglycan was also administered by local and systemic injections to mdx mice where it ameliorated many symptoms of these slightly dystrophic animals which have no dystrophin in their muscles. However, the most surprising finding was, that after systemic single injections, their normally low level of utrophin was upregulated in the treated muscle about 2.5 fold two to three weeks after the treatment.

The next step was to see if biglycan treatment improves muscle function in mdx mice. After three months of repeated systemic injections with human biglycan, the muscles of these mice without dystrophin were much more resistant to damage caused by forced lengthening and contractions.

Biglycan is present during development in humans, therefore, its use as a drug would not be expected to cause significant immune rejection. Since it acts outside the muscle cells, it does not have to cross the muscle membranes when used as a therapeutic agent. The alpha- and gamma-sarcoglycans, the two proteins to which biglycan binds, are only present in skeletal and cardiac muscles. That means that biglycan 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. It will take about 12 to 18 months to manufacture sufficient human biglycan in clinical grade

purity, so that a phase-I clinical trial could then be started in about two years with this potential Duchenne drug.

Reading through premature stop codons with PTC124.

Langdon Miller of PTC Therapeutics in South Plainfield, NJ, updated the results summarized in my reports on the Cincinnati and London conferences with the newest information about the phase-II clinical trial with PTC124.

About 13 to 15% of Duchenne patients have a nonsense or stop mutation in their dystrophin gene which changed a single amino acid codon into one of three premature termination codons, TGA, TAG and TAA. In the mRNA, these codons become UGA, UAG, and UAA and cause the protein synthesis to shut down prematurely before the new dystrophin is fully assembled. In this case, a truncated dystrophin is produced which is too short to have its normal function. PTC124 is a small-molecular drug designed to allow the protein-making machinery to *read through* the premature stop, resulting in production of the full-length protein. For a boy to benefit from this drug, the exact mutation must be determined by sequencing the region around the mutation site to see whether there really is a nonsense mutation as the cause of his Duchenne dystrophy. Only when that is true, is it possible that PTC124 could have activity. Thus, PTC124 may help the cellular machinery to overcome one of the genetic causes of Duchenne. Such a treatment is different from gene therapy or exon skipping.

PTC124 is a novel, first-in-class drug. It was found in a high-throughput automated drug screening program which tested about 800,000 compounds of low molecular weight for the ability to overcome the effects of a nonsense mutation. These compound were then optimized chemically during several years of laboratory work. Although it was the antibiotic gentamicin which was first shown to induce *readthrough* of stop codons, PTC124 is not related to gentamicin and is not an antibiotic. Details about PTC124, including its molecular structure and its intensive pre-clinical testing, have been published in *Nature*, 447, 87-91 on 3 May 2007 with a News & Views comment "Ignore the nonsense" on page 42.

PTC124 is not yet available commercially, it is still under development in animal and human studies. It is an oral medication, a white crystalline powder with a mild vanilla flavor, supplied in small foil packets. The drug is mixed with tap water, milk or juice before use.

Because PTC124 can induce readthrough of nonsense mutations in any mRNA, it is also a potential drug for other genetic disorders due to nonsense mutations, e.g., cystic fibrosis. In fact, PTC Therapeutics has already conducted several phase-II studies and hopes to initiate longer-term studies with cystic fibrosis as well as with Duchenne patients..

To determine whether PTC124 could be a useful Duchenne drug, preclinical experiments were done on cell cultures. PTC124 treatment of mdx muscle cells in these cultures showed that full-length dystrophin was produced and that it moved to its correct place underneath the muscle membrane. In living mdx mice, new dystrophin after oral dosing for up to eight weeks appeared in skeletal, diaphragm and heart muscles which reduced muscle fragility and blood CK concentrations. Toxicity studies in mice, rats and dogs given high doses of the drug have generally not shown serious, acute side effects. Inflammation of kid-

neys was observed in mice and inflammation of the adrenal gland was seen in dogs. Tumors of brown fat were found in rats in one study but were not seen again when the study was repeated. Clear evidence of any similar kidney or adrenal problems in humans has not been seen. Humans do not have much brown fat, so it is unlikely that this effect is meaningful for humans. In the clinical studies, special attention will be given to testing for kidney or adrenal gland problems.

In the first clinical testing of PTC124, an open-label phase-I trial was performed in 61 healthy 18- to 30-year old adult volunteers. This age range was chosen because it is similar to young adults with Duchenne. They received the drug three times per day for as long as two weeks. The absorption of the drug was also tested with and without food. The drug was well absorbed into the body and it was possible to maintain plasma concentrations of 2 to 10 micrograms/ml known to be active in mdx mice. PTC124 demonstrated a few side effects like nausea, diarrhea and headache at very high doses of more than 150 mg/kg. Some slight increases of liver enzymes were sometimes seen. Doses of up to 100 mg/kg/day were well tolerated by these healthy adults, a dose which is larger than those planned to be given to Duchenne boys.

These results supported the initiation of a phase-II clinical study. In the initial part of this open-label clinical trial, 26 boys with Duchenne, 5 to 13 years old, completed enrollment and testing. Six boys received a dose of 16 mg/kg/day and 20 boys received a dose of 40 mg/kg/day divided into three portions per day. The boys had different stop mutations: 15 with UGA, 6 with UAG, and 5 with UAA stop codons, distributed across exons 6 to 70. The patients were clinically evaluated for up to 21 days before the treatment, then received the drug daily for 28 days and finally had follow-up examinations during an additional 28 days. Muscle biopsies were performed before and after the treatment on the *extensor digitorum brevis* foot muscles to check for the restoration of full-length dystrophin production. Other chemical and functional tests were also done to measure the therapeutic effect and to monitor for safety.

Before the boys of the first two groups received the drug, the muscle tissue from their initial biopsy was treated with PTC124 in the laboratory. The expected dose-dependent increases in the amount of full-length dystrophin were detected in all 100% of the boys in these laboratory experiments. In about 50% of the 26 boys in both dose groups, 3 of 6 in the lower dose group and 11 of 20 in the higher dose group, a slightly visible increase in dystrophin could be detected in the biopsy material obtained at the end of treatment with the drug for 28 days. One of the reasons why new dystrophin was not found in all boys may be that the PTC124 concentration in the blood was below the intended concentration range of 2 to 10 micrograms/ml of plasma, possibly due to the faster degradation of the drug in children when compared to adults. But all these boys showed a reduction of the CK activities during the treatment which then increased again during the follow-up period without treatment as expected for a pharmaceutical agent that has to be continuously administered. Some mild to moderate but infrequent side effects were observed like diarrhea, nausea and headache, and there were no clinically relevant laboratory abnormalities.

Several parents and teachers observed that, after the

treatment, some of the boys showed greater activity, increased endurance, and less fatigue than before the treatment. These are only informal parent reports, so these types of findings need to be evaluated in more detail.

Based on these results, it was assumed that a higher dose of PTC124 could lead to the appearance of new dystrophin in sufficient quantities to have a therapeutic effect. Therefore 12 additional boys with Duchenne were enrolled into this trial. These boys received 80 mg/kg/day PTC124 divided into three separate doses and given for 28 days. This dose should be sufficient to reach a concentration of at least 2 to 10 micrograms/ml in the blood plasma. This extension of the trial has been completed, the data are now being analyzed, and the final results will be available soon.

At the end of his presentation, Dr. Miller mentioned three questions which have to be answered before PTC124 can become a drug for Duchenne boys: (1) Will the short-term positive activities of PTC124 translate into the desired long-term clinical benefits like increased muscle function, strength, and endurance as well as better quality of life? (2) What dose of PTC124 will most safely provide the best clinical benefit? (3) How many months will it take to translate the short-term pharmacological activity into longer-term clinical benefit?

Only a long-term, controlled clinical trial can address these issues. The next steps to be undertaken by PTC Therapeutics thus are to finish the analysis of the data of the completed phase-IIa studies and then to prepare all the requirements for the review of a longer-term clinical trial by the American FDA and the European EMEA before the end of 2007. This longer trial is expected to start in the coming months. PTC will also open a longer-term extension study for the boys treated in the phase-IIa trial.

Project Catalyst: Finding and developing four new drugs to treat Duchenne dystrophy. The company PTC Therapeutics in South Plainfield, NJ, is focused on the discovery and development of small-molecular drugs that modify the *post-transcriptional control* (PTC) of the biosynthesis of proteins *after* the genetic information is copied, transcribed, into RNA from the gene. *Ellen Welch*, a group leader of this company, introduced *Project Catalyst*, a program for finding and developing small chemical compounds that could become drugs for a therapy for Duchenne muscular dystrophy.

Project Catalyst was started in May 2004 with the aim to identify with automatic screening methods among several hundred thousand compounds those that could modify the production, their *expression*, of four biological targets in muscle cells. These four targets were selected, because research had already shown that the up- or downregulation of their expression could maintain and improve muscle structure and function in Duchenne patients: The decrease or downregulation of *myostatin* and the upregulation of the muscle specific *insulin-like growth factor* IGF-1, but not of the liver specific isoform, would promote muscle growth and regeneration. The increase or upregulation of *utrophin* and *alpha7-integrin* would stabilize the muscle membrane and thus improve muscle function.

The automatic screening methods for finding these potential Duchenne drugs use a newly developed test procedure to measure their activities with a reporter-protein system: the mRNAs for each of the four targets were com-

bined with the gene for the enzyme luciferase from fireflies. In the presence of an active compound, the light intensity produced by the luciferase would then be either increased or decreased. Measuring precisely and automatically light intensities is much easier than analyzing the biological effects of the targets.

A relatively small number of compounds with at least some of the desired properties are now being optimized by varying their chemical structure and characterizing their biological, pharmacological and medical properties. Changes of the chemical structure which affects, e.g., activity, potency, solubility, permeability, metabolism, toxicity, side effects, oral bioavailability, and other properties, are ongoing. This optimization process occupies many chemists for several years. Finally, for the most promising compounds, laboratory synthesis has to be scaled up sufficiently to produce enough material for pre-clinical testing in isolated muscle tissue and in living animals like mdx mice. A number of compounds with the ability to modify the expression of the targets are now in various stages of this optimization process.

PTC Therapeutics has now several potential drugs available that downregulate myostatin at very low concentrations, upregulate the muscle isoform of IGF-1, but not the liver specific one, upregulate utrophin and alpha7-integrin up to twofold. These very promising potential drugs will be further optimized during the next years with the aim to start phase-I clinical studies for toxicity with Duchenne boys in about 2009.

The information of the dystrophin gene itself, including its mutations, will not be changed by these drugs, they will probably have to be taken for the life-time of the patient. But they could possibly later be used in combination with each other and with other pharmaceutical drugs and genetic techniques like exon skipping for providing an optimal and highly effective Duchenne therapy.

Inhibition of NFκB improves muscle structure and function. *George Carlson* and his team at Still University in Kirksville, Missouri, were trying to understand why the loss of dystrophin leads to a muscular dystrophy in the mdx mouse, because they wished to design a targeted research approach for a therapy. It had been proposed that the entry, the influx, of increased amounts of calcium ions, charged calcium atoms, into the muscle cells was responsible for the dystrophic symptoms. But the measurement of the calcium influx into isolated severely dystrophic fibers from the expiratory *triangularis sterni*, TS, muscle of mdx mice showed that this influx was not really increased in these stressed fibers compared to the influx into normal muscle fibers under the same conditions. This indicated that an increase of calcium influx is not responsible for muscle degeneration in muscular dystrophy. However, the TS muscle is passively stretched every time the animal breathes in, suggesting that passive stretch of dystrophic fibers activates a mechanism that promotes muscle degeneration. Other investigators showed that passive stretch activated the NFκB signaling pathway in muscle. *Dr. Carlson* and his colleagues soon began testing the hypothesis that activation of this pathway was responsible for the damage to the stretched dystrophic TS muscle.

This protein NFκB was found in 1986, in the same year as the dystrophin gene. It is present in the cytoplasm of all

cells, but most of the time, it is inactivated there by another protein, the inhibitory κ B, or I κ B. When an inflammation is initiated by rather complex regulatory processes to combat a viral or bacterial infect, the I κ B kinase complex, IKK, adds two phosphate groups to the NF κ B and thus frees it from its inhibitor. The now activated NF κ B migrates from the cytoplasm into the cell nucleus and, because it binds there to the promoter and enhancer regions of many genes, it causes the upregulation of these genes. Several hundred genes are known who are part of the different chains of reaction steps, co-called *cascades*, which depend on the active NF κ B in the cell nucleus. When the inflammation is not needed anymore, the activation of NF κ B is stopped by anti-inflammatory factors. If genetic mutations, other pathogenic or stress situations do not allow these factors to deactivate the NF κ B, some chronic diseases develop like arteriosclerosis, lung fibrosis, asthma, rheumatic arthritis, and probably also Duchenne dystrophy.

Dr. Carlson began testing drugs that inhibit the activation of NF κ B and studied the effects of these drugs on the chronically stretched TS muscle. There are a number of drugs which can inhibit the activation of NF κ B. One of these is pyrrolidine dithiocarbamate which has been shown to prevent the split of the inactive NF κ B-I κ B complex so that NF κ B cannot move into the cell nucleus. This drug was found to be effective in improving the structure and function of dystrophic muscle. Some of these NF κ B inhibiting drugs are already approved on the market for the treatment of other conditions. One of these is Sulfasalazine, which is used for the treatment of juvenile rheumatoid arthritis.

Dr. Carlson and his coworkers have already tested these two drugs on their isolated TS muscle fibers from mdx mice and found that their diameter and their function had significantly increased. At the present time, Dr. Carlson is screening several drugs which inhibit the NF κ B pathway and is particularly interested in testing compounds that are currently being used to treat other conditions. At the end, Dr. Carlson said: "Maybe we should start considering clinical trials for some of these NF κ B inhibiting drugs that are currently being used to treat other human diseases."

Beta-agonists, potential drugs for Duchenne dystrophy. *Gordon Lynch* of the University of Melbourne in Australia presented the research work of his laboratory to use the very well known drugs called *beta-agonists* to maintain and increase the muscles of Duchenne patients. Beta-agonists are hormone-like substances that bind to specific receptor proteins in the cell membranes and then influence their activity to start *signaling pathways* inside the cell, by which, through a chain of interacting specialized proteins, chemical signals are sent to biological targets in the cell which have to be activated, inhibited, or otherwise modified to ensure the proper functioning of the cells under changing conditions. The pathway, the *reaction cascade*, influenced and modified by beta-agonists is the so-called *beta-adrenergic signaling pathway* which is, among other biological effects, important for controlling protein synthesis and protein degradation.

Some beta-agonists are drugs which are already widely used by inhalation to relax the airway smooth muscles of asthma patients, they are *bronchodilators*. Others have

powerful anabolic effects on skeletal muscles, especially when injected at higher doses into the blood circulation. They are sometimes illegally exploited by athletes seeking improvements in muscle size and reductions in body fat.

As beta-agonists may also be useful to reverse the muscle wasting in elderly people, Dr. *Lynch* and his colleagues tested their anabolic potential in experiments with 28-month-old rats, which were approaching the end of their usual lifespan of about 30-32 months. In 2004, the researchers showed that daily treatment of these old rats for 4 weeks with 1.4 mg/kg *fenoterol* by intraperitoneal injection, into the abdomen, reversed age-related muscle wasting. In comparison with 16-month-old adult rats, that had completed their growth, the muscle mass of the old rats was significantly larger, the muscle fibers had a larger diameter, the strength of the muscle was increased, and many of the slow muscle fibers had become fast fibers which led to faster and stronger muscle contractions. These results indicated that beta-agonists could possibly be used to treat *sarcopenia*, the muscle wasting and associated weakness in the growing population of elderly people. Fenoterol is one of the older generation of beta-agonists, and in subsequent experiments Dr. Lynch and his colleagues have examined the efficacy of more powerful, new-generation beta-agonists, formoterol and salmeterol, both of which are approved for treating asthma in humans.

If muscle wasting of healthy elderly people could be treated by beta-agonists, then it is obvious that they also could be beneficial for patients with muscle wasting diseases like Duchenne muscular dystrophy.

In fact, a first clinical trial with Duchenne and Becker dystrophy patients has already been performed. As fenoterol could not be used, the participants were treated for 28 weeks with 8 mg/day *albuterol*, another beta-agonist which is an approved drug. This low dose was chosen after another one-year trial with adult FSH dystrophy patients had shown that at doses of 16 and 32 mg/day, albuterol led to some unacceptable heart problems. The reduced dose in the Duchenne trial did not cause any side effects but it produced only a modest increase of muscle strength which was insufficient to have a therapeutic effect.

Therefore, Dr. Lynch and his colleagues asked whether the more powerful beta-agonist, *formoterol*, would be effective in dystrophic mdx mice. Very low doses of formoterol, 25 micrograms/kg, increased the muscle fiber size and improved the function of two tested skeletal muscles of these dystrophic mice. Importantly, these improvements in muscle strength were not associated with any changes in muscle fatigue.

The plans for future research towards a Duchenne therapy will include further pre-clinical experiments, especially to separate the positive effects of the beta-agonists on skeletal muscles from their potentially negative influence on the heart. Since these beta-agonists act on the same types of receptors that are found in the skeletal muscles and the heart, separating their effects is an important scientific challenge. After all, Duchenne patients do not need an enlargement of their hearts. Also, another side effect, the downregulation of the beta receptors in the muscle cell membranes should be avoided to allow the development of beta-agonists with long-term effects. After these problems have been solved in mice and dogs, it will be possible to perform new clinical trials with Duchenne boys.

Blocking inflammatory agents. The degradation and death of muscle cells in muscular dystrophy 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.

Melissa Spencer of the University of California in Los Angeles reported on experiments to counteract inflammation and immune response and thus to find ways to eventually replace the steroids with drugs that target specific immune mediated damage. Some of the following text is based on the presentation of *Sylvia Lopez*, a graduate student in Dr. Spencer's laboratory, at last year's PPMD meeting in Cincinnati.

Studies have shown that increased levels of the CD4 and CD8 T cells and more importantly, myeloid cells, of the immune system accelerate the progression of the disease. These cells secrete cytokines, molecules that promote inflammation and the development of fibrosis, in mdx and Duchenne muscles. In healthy people, this is a normal process of wound healing which stabilizes weak tissue and promotes healing. In Duchenne boys, this process gets out of control and becomes chronic so it is like a continuously healing wound. Therefore, inhibition or removal of immune cells and also the modulation of active cytokines would possibly slow down the degradation and fibrosis of dystrophic muscles. A number of approved anti-inflammatory drugs already exist. If they could be shown to positively influence Duchenne dystrophy, it would take much less time to obtain FDA permission for the additional treatment of Duchenne than for the approval of a completely new drug..

Three of these drugs are now being tested in Dr. Spencer's laboratory with mdx mice. They are already being tested in clinical trials on patients with other diseases: *CTLA-4Ig* against rheumatoid arthritis, *Galectin-1* also against arthritis, it has already been shown to improve muscle regeneration, and *Anti-asialo GMI*, an antibody used in Parkinson's disease. Testing in mdx mice of two other drugs will follow; *Remicade*® and *Enbrel*®, both approved for rheumatoid arthritis and other diseases. Another important target for an anti-inflammatory drug is the protein NF-kappa-B, which was discussed at this meeting by Dr. *Carlson*.

At the end of her presentation, Dr. *Spencer* mentioned important new results. *Osteopontin* is a protein which gets into the blood stream and which has many functions in bone biology, immune regulation, cell survival, inflammation and cancer metastasis. It has been shown that its concentration is increased in the blood and also in the muscles of mdx mice. Mdx mice without osteopontin have better muscle strength, lower CK values, and reduced fibrosis. Thus, if a drug could be found which would inhibit osteopontin in Duchenne patients, it would probably improve muscle repair, improve their function, and decrease fibrosis.

Long-term treatment studies will be necessary to estab-

lish whether these drugs could become therapies for Duchenne patients and thus could be used instead of steroids and possibly in combination with genetic methods like exon skipping to improve their life expectancy and quality.

Clinical trial with MYO-029 to inhibit 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 limits the growth of muscles. Therefore, by inactivating myostatin, it should be possible to stimulate the regeneration of the muscle fibers of Duchenne boys so that they would not be destroyed as fast or might even increase in size.

Non-dystrophic mice whose gene for myostatin had been deactivated 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. Recently it was discovered that in the so-called *bully whippets*, English racing dogs, their extremely enlarged muscles were also caused by the absence of myostatin. And in Berlin, a now 8-year old physically very strong *boy* was identified whose skeletal muscles are about twice as large as those of a normal child. This is a strong indication that the downregulation or inhibition of myostatin would lead to an increase of muscle growth in Duchenne boys, too. However, a myostatin therapy would not be able to influence the genetic cause of the disease, but it could probably, in combination with the more basic genetic treatments like dystrophin gene transfer and exon skipping, enhance their therapeutic effects.

Kathryn Wagner of the Wellstone Muscular Dystrophy Center at the Johns Hopkins University in Baltimore reported already at the meeting in Cincinnati 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.

To answer the question whether the absence of myostatin would have similar effects on the heart, further 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 in Collegeville near Philadelphia a clinical trial with *Myo 029*, a specific antibody which binds to myostatin and blocks its activity, is now being performed. This protein does not cause immune rejection because its structure is the human one, it is "humanized". It can be injected into the blood circulation or under the skin.

Three groups of 36 adult muscular dystrophy patients each, among them some Becker patients, are being treated intravenously with 1, 3, and 10 mg/kg MYO-029 every two weeks for 24 weeks, followed by 12 weeks of tests and clinical supervision.

The aim of this phase-I/II clinical trial is to assess safety and prove that there is some efficacy. Preliminary results show that MYO-029 is well tolerated and that its

short-term safety is excellent. There will be a publication as soon as the trial is completed and the results fully analyzed and evaluated.

As it will be important to know the long-term effects of this drug, a study with dystrophic dogs is planned. Wyeth is not the only company active in this field. Other companies are developing other methods to block the activity of myostatin, not only for the treatment of muscular dystrophies but also for other, much more economically important health problems like the muscle deterioration in older people.

Dr. Wagner finished her presentation in Cincinnati with the warning, repeated here: The 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.

BBIC inhibits proteases, but Myodur (C101) does not.

Lee Sweeney of the University of Pennsylvania in Philadelphia discussed new research on the inhibition of proteases. The degradation of muscle proteins in Duchenne dystrophy is caused by several different proteases, protein-destroying enzymes, among them the enzyme *calpain*, which is activated by calcium, and a large protein 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. Researchers are trying to inhibit the activity of calpain and other proteases and thus delay muscle cell degradation.

One of these inhibitors is the *Bowman-Birk inhibitor concentrate*, BBIC, a natural protein composed of 71 amino acids, which can be isolated from soybeans. It is a water-soluble substance that can be orally taken. As it is too big to enter the muscle cells, it blocks several proteases like the digestive enzymes like trypsin and chymotrypsin outside the cells and interrupts signaling pathways that can produce inflammation processes in Duchenne dystrophy. BBIC blocks the dystrophic process in mdx mice by inhibiting the muscle degradation, this action is comparable to prednisone. Long-term treatment with BBIC increases the muscle mass and strength in mdx mice. CK activities are reduced considerably and fibrosis also. From other applications in cancer patients, it is known that BBIC is a very safe drug.

Clinical phase-I trials are now being prepared together with Dr. *Kenneth Fishbeck* at the National Institutes of Health, NIH, in Bethesda near Washington and will start as soon as FDA approval is obtained. If the clinical trials show that similar results as those found with mdx mice can be obtained in Duchenne boys, this rather benign drug, although it cannot solve the basic genetic problem, can possibly slow down the muscle degradation in Duchenne Dystrophy. Soybeans contain other proteases also, so BBIC must be isolated and purified from them. Eating the beans directly has no effect.

The tripeptide *leupeptin* was the first inhibitor identified that possibly could reduce the activity of the enzyme calpain in mdx mice. Leupeptin consists of three amino acids with a chemically reactive aldehyde group which is

essential for the inhibitory activity. *Myodur*, the investigational compound C101 developed by the company CepTor in Baltimore, is a combination of leupeptin with carnitine which was thought to be able to inhibit calpain more effectively than leupeptin as reported by Dr. *Theresa Michele* of CepTor at the 2006 PPMD meeting in Cincinnati. In the laboratory of Dr. *Sweeney*, further pre-clinical studies were performed to check the effects of leupeptin as well as of Myodur before clinical studies could be undertaken. The results were disappointing: in experiments with mdx mice, it was shown that Myodur after a treatment for 8 weeks or leupeptin after a treatment for 6 months did not increase the muscle size, did not improve the muscle function, and did not reduce muscle damage. In effect, calpain is not inhibited but upregulated by these drugs, and calpastatin, a natural inhibitor of calpain, is downregulated. So calpain does not appear to be a useful target for a Duchenne drug. The clinical trial planned by CepTor will not take place.

International clinical trials with pharmacological

agents: The *Cooperative International Neuromuscular Research Group*, CINRG, a cooperation of 22 hospitals in the US, in Canada, Belgium, Italy, Sweden., Argentina, Australia, and India, performs clinical trials on Duchenne boys. *Diana Escolar*, of the Children's National Medical Center in Washington, DC, who organizes and supervises these trials, discussed the most recent completed and still ongoing trials. For the documentation of these studies, standardized control methods have been developed to measure not only muscle functions but also many other parameters including the quality of life.

Dr. *Escolar* did not discuss the results of two recently completed trials which have already been published. They are the trial with *creatine and glutamine*, published in the *Annals of Neurology* 58:151-155 (2005), which showed a slight but not significant effect on muscle function, and the trial with *oxatomide*, published in the *European Journal of Pediatric Neurology*, April 23, 2007, which did not show a significant improvement of muscle function.

Dr. *Escolar* then presented the first results of an open-label phase-I/II pilot study of *pentoxifylline* in Duchenne boys who did not receive steroids for at least one year before enrolment. Seventeen Duchenne boys aged 4 to 7 years participated. The trial started in March 2002 and was completed in July 2006. At the beginning, each boy was clinically controlled for 3 months and then treated for 12 months. The effect of the treatment was measured by quantitative muscle function tests and by a number of other tests. Eight of the boys left the study prematurely, some of them because of side effects like leukopenia, a decrease of the number of the white blood cells.

The most important, however negative, result was that one year treatment with pentoxifylline *failed to increase* significantly muscle strength and function. But, although the boys did not receive any steroids, there was *no deterioration* of their muscle strength. Thus there might have been a stabilizing effect of the treatment. To check whether this is really the case, a larger and controlled study became necessary.

Such a double-blind study with 64 Duchenne patients older than 7 years but still able to walk at least 10 meters has been started in September 2005, it will be completed in December 2007. Each boy receives the drug or a placebo

daily for 12 months. The patients can be on steroids or other additional compounds like creatine, glutamine, coenzyme Q10 etc. but are not allowed to change these treatments during the study.

A phase-II open-label pilot study with *coenzyme Q10* on 13 steroid-treated ambulant 7-to-11-year old Duchenne boys started in September 2001 and was completed in January 2005. The initial dose of 90 mg/day was raised to 400 mg/day which lead to the targeted serum level of at least 2.5 microgram/ml. The only side effect was migraine in one boy on a high dose which could be resolved with dose reduction. The boys were treated daily for six months and then had the option to remain on the drug until the completion of the study.

This treatment with coenzyme Q10 *increased the muscle function* by 7.3% on the average in these steroid-treated Duchenne patients. The muscle function was measured by total quantitative muscle tests.

Based on this result, a randomized placebo-controlled clinical trial with 120 non-ambulatory 10-to-15 year old patients was started in March 2006 which is expected to be completed in December 2008. The patients are being treated daily for 12 months in 4 groups receiving either 0.75 mg/kg/day prednisone, or coenzyme Q10 to get a level of 2.5 microgram/ml serum, or a combination of both drugs.

How does the FDA approve a Duchenne drug?

Tan Nguyen from the FDA Office of Orphan Products Development in Washington explained in a very engaged and optimistic presentation that the Food and Drug Administration, FDA, is not there to delay or stop the development of much needed drugs, but that the concerns and the anxiety of a family with a child having an incurable disease are well understood by the FDA. Much has been done to accelerate the approval process of drugs for rare and serious diseases like Duchenne muscular dystrophy, and to lessen the financial burden associated with their development.

The original Federal Food, Drug, and Cosmetic Act was signed into law in 1938 after the elixir sulfanilamide disaster caused the death of 107 patients in the United States. But until 1962, the FDA could only force the manufacturers to prove that their drugs were safe. After more than 10,000 deformed children were born in Germany by thalidomide, trade named *Contergan*, an even larger catastrophe was barely avoided in the US by the delay its approval because of insufficient safety data. The drug approval process of the FDA was radically altered in October 1962 with the introduction of the Kefauver-Harris amendments. From that time on, manufacturers were required not only to prove that a drug is safe but also that it is effective for FDA to approve it for marketing.

A non-orphan drug development has to go through the following stages: (1) The pre-clinical phase involving laboratory and animal studies taking on average 6.5 years at the cost of \$1-12 million to assess its safety, biological activity, and formulations; (2) the *phase-I* clinical investigation on 20-100 healthy volunteers (duration 1.5 years on average) to determine its safety profile in humans; (3) the *phase-II* clinical investigation on 100-500 patients (duration 2 years on average) to evaluate optimal dosages, safe-

The treatment results are compared with patients receiving an improved standard of care, but no placebo is used.

The results of all trials will be published as soon as they are fully evaluated. Information on these and other Duchenne trials are on the internet at www.clinicaltrials.gov.

At the end of her presentation, Dr. Escolar showed a list of what she, as the director of the neuromuscular clinic at Children's National Medical Center suggests the parents should give their Duchenne boys:

Treatment with *prednisone* 0.75 mg/kg/day, or *deflazacort* 0.9 mg/kg/day only if the patient or the family is obese and after nutritional counseling, should start immediately after diagnosis; *vitamin D* and *calcium* supplements; *strict diet* based on high protein, low hydrocarbons and fats, i.e. lean meat, fresh fruits and vegetables; *creatine* 5 g/day; glutamine 0.3 g/kg twice a day; *coenzyme Q10*, 200-400 mg/day; other accepted, but not encouraged supplements, depending on parents, are e.g. green tea extract, arginine, and anti-oxidants like Protandim. When the steroid treatment is started at 2 to 4 years of age, the weight gain is less a problem than when started later. Decreased growth is another side effect at all ages. Behavioral changes are less or transient when the treatment is started early.

ty, and efficacy; (4) the *phase-III* clinical investigation on 1,000-5,000 patients (duration 3.5 years on average) to confirm the drug's safety and effectiveness of long-term use. The cost of all three clinical investigational phases is approximately \$15-300 million. Therefore, the total development time may be up to 15 years at an average cost of about \$360 million. Generally, of 5,000 compounds evaluated, only one is approved and reaches the market. For the development of an *orphan drug* for a disease like Duchenne muscular dystrophy with a limited number of patients, some of the investigational requirements may not be applicable. Thus, they have to be creatively modified within a rigid regulatory framework.

When the pre-clinical studies have been completed, the drug sponsor submits an *Investigational New Drug Application*, IND, with the FDA to obtain permission to conduct clinical studies of the drug. After the completion of all phases of clinical investigation, the sponsor files a *New Drug Application*, NDA, to start the final marketing approval process. The FDA approval process involves reviewing pre-clinical and clinical data to support the safety and effectiveness of the drug. This takes, in most cases, one year or less at the cost of more than a million dollars.

A drug is called an *orphan drug* if, among other definitions, it is intended to treat a rare disease affecting less than 200,000 people in the United States. With about 10,000 Duchenne boys living in the US, this disease is considered a rare disease and a drug developed for its treatment can be considered an orphan drug. Six potential Duchenne drugs have already received orphan-drug designation: mazindol, oxandrolone, PTC124, 2'-O-methyl AONs for exon skipping, leupeptin, and idebenone. Unfortunately, some of these drugs are no longer in active clinical development for various reasons. So far, the FDA has

approved over 300 orphan drugs for about 180 other rare diseases.

When a company develops an orphan drug, it gets a number of financial incentives through the orphan-drug designation process. These are: (1) 50% of the clinical development expenses are credited towards its federal income tax; (2) waiver of the normal FDA marketing application fee of over a million dollars; and (3) a seven-year marketing exclusivity following its marketing approval. The company may also compete for orphan drug grants of up to \$400,000 a year for up to four years (\$1.6 million total) to defray the costs of clinical trials. It should be pointed out that a potentially shorter time to approval by up to three years in some cases may be possible through the *Subpart E approval*. This is mainly achieved by approving the drug on the basis of adequate data from phase-II clinical investigations. A still unapproved drug under active investigation but shown to be promisingly safe and effective may be allowed to be given to patients with a serious or immediately life-threatening illness under the compassionate use program if no satisfactory alternative therapy exists.

Dr. Nguyen also discussed many details of how the FDA oversees the entire development process in order to assure the safety of patients in clinical trials and the demonstration of the drug's effectiveness.

Orphan drugs, too, have to be safe and effective.

Among the many answers Dr. Nguyen had to give to questions after his presentation, were the following two:

"It is unclear to predict with any reasonable certainty at this time whether every one of the many antisense oligonucleotides with different sequences would have to undergo the entire approval process as new drugs. It would be nice if only the first one of each kind would have to go through it, and that for the following ones, the process

would be somewhat abbreviated. It is reasonable that the Duchenne community should do some forward thinking on this issue and provide appropriate suggestions or recommendations in some public forum."

"The FDA insists that the approval of an orphan drug is subject to the same high standards for safety and efficacy as for any non-orphan drug. Even so, the FDA is sensitive to some parents' opinion that since the disease is so severe, they would rather give an unapproved but promising drug to their sick sons immediately and not wait many years more, even if this would increase the risk considerably and that something negative could happen."

Kate Bushby said in this context and I quote her words in almost their entirety: "We know that we have to get something working to the patients fast. But we have to minimize all the risks. I don't want to give something to a 6-year old boy which is possibly not effective, because Duchenne does not kill 6-year old boys tomorrow. One does not learn enough from a phase-I trial to extrapolate to a long-term treatment. Each stage of a trial tells you something different. At each stage, a drug might fail, we don't want that, but it may. There might even be fatalities! If a doctor gives a drug after a phase-I trial and it is shown to be dangerous in the phase-II trial, he will be in legal trouble. If you want the drug to go to a wide community, it has to go through the regulation process. Only then will it be available and affordable to all who need it. If you do shortcuts and go down the wrong route, you will delay the problems. One should be able to take the time and get all the necessary data together. It would be very bad, if a drug, approved after a short-term trial, shows to be unsafe during a long-term treatment, then it has to be withdrawn. And parents cannot accept the responsibility and just sign a waiver. That would be unethical. And what happens, if your son dies? We have the responsibility for the patients and for the parents, too."

TREAT-NMD, a Network of Excellence of the European Union.

Serge Braun, the Scientific Director of the French muscular dystrophy association AFM, Association Française contre les Myopathies, first described this very active national patient organization. It was founded 1958, has now 75 clinical centers in France and is known throughout the country by its Téléthon, a 30-hour television program on the first weekend of December of each year. After it was started in 1987 with the help of *Jerry Lewis* and modelled on the US Telethon, this fund-raising event alone provides on average 140 million dollars every year. The entire budget of the association for 2006 was 170 million dollars of which 88 million were used to fund about 400 research projects for the therapy of neuromuscular diseases, among them were about 100 projects outside France including the United States.

Dr. *Braun* then explained how the AFM and other patient organizations in Europe became aware of how the research activities for therapies, diagnostics, and patient care for neuromuscular diseases in the different countries of the European Union were poorly coordinated, an unacceptable situation in view of the severe consequences of these rare diseases. The AFM member *Françoise Salama*, who has a son with Duchenne, together with many of her European

colleagues lobbied the European Commission to provide funding for integration of activities in this area, and this was very successful, with a call for networks of excellence in this area appearing in 2004. The aim of such networks is "to strengthen scientific and technological excellence on a particular research topic by integrating at the European level the critical mass of resources and expertise needed at creating a progressive and durable integration of the research capacities of the network partners". Following a long application process, this program, TREAT-NMD, was approved in December 2006 with a budget of 10 million euros - about 15 million US dollars - for 5 years, and active work was started in January 2007.

TREAT-NMD is now *a network of excellence that will share expertise between basic and clinical academics and industrial partners in order to develop technological and methodological tools with a view to accelerate the elaboration of new therapies for neuromuscular diseases.*

TREAT-NMD has its offices at the University of Newcastle in England, it is coordinated by *Kate Bushby* and *Volker Straub*, and has a Science and Technological Advisory Council with *Marianne de Visser* of the University Amsterdam in the chair, and a website: www.treat-nmd.eu,

which, among a great amount of information, also contains a frequently published newsletter that is being sent to more than 1,000 people whose addresses are on its mailing list.

This introduction by Dr. Braun was followed by the presentation of *Kate Bushby* with the title *TREAT-NMD in action, the first six months*. The most important aims of TREAT-NMD are: (1) To further international cooperation focussed on the development of therapies for Duchenne and congenital muscular dystrophies as well as for spinal muscular atrophy; (2) to promote multiple therapeutic approaches by suggesting and coordinating clinical trials; (3) to find patients to participate in the trials by the creation of an international data bank; (4) to create standards for the clinical trials thus allowing their results being compared with other similar trials; (5) to develop internationally accepted standards for diagnostics and care, and (6) to distribute up-to-date knowledge about research, diagnosis and care to all patients, their families, and the treatment centers especially to those in the less privileged areas of the world.

TREAT-NMD has 21 active partners - universities, patient groups, government agencies, biotech companies, and individuals - across Europe who are primarily responsible for delivering the aims of the network. It works together with groups from all over the world, and new collaborations in this important area are welcome.

Dr. Bushby then mentioned four examples of present and future activities:

(1) Setting up an international patient registry in partnership with existing registries, so that there is a standardized minimum set of clinical and diagnostic information of

patients to be used for the development and future management of a supranational data bank. This would allow to identify suitable patients for clinical trials, the collection, monitoring and comparison for research of long-term clinical data of patients undergoing different treatments, and for the information of individual families about advances of research and care.

(2) Establishing the most advanced standards of care in cooperation with the Center of Disease Control and Prevention, CDC, in Atlanta in the United States. Work on this project has already begun, it will cover all aspects of medical and social management of the patients. Ways will have to be found to make sure that the recommendations will be followed in all treatment centers and partnership with patient organisations will be a key way to make this happen..

(3) Standardizing clinical outcome measures for trials, that is, the way clinical changes have to be measured. Two workshops were already held in May and June 2007.

(4) A Clinical Trials Coordination Center, CTCC, has been established at the University of Freiburg in Germany which will develop recommendations for the design of clinical trials for rare diseases in cooperation with pharmaceutical companies and the regulatory authorities.

At the end of her presentation, Dr. Bushby said that TREAT-NMD is a great opportunity for the entire Duchenne community to move forward, and that *TREAT-NMD is already in action*. The office in Newcastle can be reached by e-mail: info@treat-nmd.eu and is always very happy to receive enquiries and comments.

Why do we need to know the exact mutation?

Robert Weiss of the University of Utah in Salt Lake City answered this question, as did *Kevin Flanigan* at the 2006 meeting in Cincinnati. The following text uses the summary of Dr. Flanigan's presentation as the basis with some new information added.

The mutation of a boy who is suspected to have Duchenne muscular dystrophy should be identified to confirm that he really has Duchenne, and not some other muscle disease, e.g. one of the many limb girdle dystrophies which might show similar symptoms. If the mutation shows that the reading frame of the dystrophin mRNA is shifted, then a diagnosis of Duchenne muscular dystrophy is very likely, whereas a mutation which keeps the reading frame intact, predicts in most cases Becker muscular dystrophy. Knowing the exact mutation allows improved genetic counseling of the boy's family and his maternal relatives, among whom Duchenne carriers can be detected. Finally, some potential new therapies, such as exon-skipping, and stop-codon readthrough with PTC124 require detailed knowledge of the mutation in the patient's dystrophin gene, they are *mutation specific*.

In order to detect deletions and duplications, the analytical technique now widely used is the *multiplex ligation-dependent probe amplification* method, MLPA, developed by Dr. *Jan Schouten* of the company MRC-Holland in Amsterdam. For this as for other genetic tests, only 5 to 10 ml of full EDTA blood of the patient is necessary from which the genetic material is isolated from the white blood cells. The patient does not have to come to the laboratory,

the blood sample can be sent by mail.

To give a very short description of the procedure: 158 oligonucleotides 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 oligonucleotides designed for its particular sequence bind on two sites and then are connected, ligated, with an enzyme to each other. The ligated nucleotides serve as a template for a multiplication of the exon by the *polymerase chain reaction*, PCR, a very powerful amplification 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 oligonucleotides for this exon cannot bind to the exon sequence and thus cannot join each other, so the corresponding peak is missing in the chart.

This technique detects deletions and most duplications of all 79 exons of the dystrophin gene in Duchenne patients, but does not detect point mutations. Because it is a *quantitative method*, deletions and duplications may be 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 this method.

However, positive results, that indicate a deletion of a single exon when there is none, may occur in the rare event of a polymorphism or mutation 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 may have a point mutation. With the *single condition amplification/internal primer* sequencing technique, SCAIP, developed in Dr. Flanigan's laboratory, it is possible to find and characterize point mutations in detail, including premature 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 with one single *polymerase chain reaction*, PCR, and then directly sequenced using standard automatic gene-sequencing methods to detect the point and other mutations.

The newest method of highly effective and accurate genetic analyses of the dystrophin gene is available using new microarray based platforms, referred to as *gene-chip technology*. In effect, as described in the next chapter about the CETT program, such a gene-chip test for large deletions and duplications, and small point mutations, is now available at Emory University in Atlanta. It is planned that gene-chip based methods will be offered by an international network of clinical laboratories,

With these test methods, MLPA, SCAIP, and gene-chip tests, 95-98% of all mutations can be found. However, the remaining 2 to 5% may be missed. If the patient has definite Duchenne or Becker symptoms, but no detectable mutation in 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

protein detection methods. The *western blot* technique has the advantage of giving an indication of the amount and the size of the dystrophin protein, but the *immunofluorescence* technique is mostly used because dystrophin, when it is present, can be made clearly visible under the microscope. The cell membranes of muscle fibers in a muscle biopsy sample from a Duchenne patient remain dark, those from a muscle-healthy person show uninterrupted bright lines of fluorescent light, and in those from a Becker patient only some segments of bright fluorescent light are visible.

Dr. Johan den Dunnen of Leiden University Medical Center sent a message saying that DNA data are not sufficient to conclude what really happens at the level of the mRNA and thus it is not possible to decide which AON to use for an exon-skipping therapy. Published data show that in 5 to 10% of Duchenne patients with deletions or duplications the consequences are not as expected. Thus before such a treatment is started, mRNA should be isolated from a muscle or skin biopsy and sequenced around the deletion or duplication breakpoints to confirm what has been predicted by the DNA tests. This check has been performed for all Duchenne boys who participated in the first exon skipping clinical trial in the Netherlands. Furthermore, the cultured cells were used to prove that the effect of the exon skipping treatment was as desired, i.e., that the last nucleotide before the deleted exon or exons was connected with the first nucleotide after the deletion without a shift of the reading frame. Messenger RNA can also be used to find the rare mutations which cause incorporation of intronic fragments among the exons in the spliced dystrophin mRNA.

A new era of genetic testing for Duchenne muscular dystrophy has begun.

CETT (Collaboration, Education and Test Translation Program). This program was presented by *Madhuri Hegde* of the Emory University School of Medicine in Atlanta, Georgia. This project is a joint development supported by the Centers for Disease Control and Prevention in Atlanta, the Office of Rare Disorders of the National Institutes of Health in Bethesda, Emory University in Atlanta, the American Society of Human Genetics, the American College of Medical Genetics, the Society for Inherited Metabolic Disorders, and the Genetic Alliance. It was started in 2006 with the aim to change the unsatisfactory translation of research genetic testing to clinical laboratories for rare diseases (1) by encouraging clinical and research collaboration for the development of high quality genetic tests, (2) by supporting the collection of genetic and clinical data in public databases to promote therapeutic research, (3) by developing guidelines for laboratory testing, and (4) by providing and distributing up-to-date information material for patients, their families and their medical and other caregivers. Details of CETT can be seen on the internet at www.cettprogram.org.

Duchenne muscular dystrophy was one of the first groups to use the CETT program model to develop a collaboration between clinical, research, and patient advocacy interests. Through the efforts of many advocates like *Jerry Lewis* of the MDA in the past and of *Patricia Furlong* of

the PPMD at present, the problems and needs of the about 10,000 Duchenne boys in the United States are not only known by the general public but also among the politicians in Washington and the state capitals. The CETT-PPMD collaboration has already reached its first important goal, namely the validation and implementation of microarray-based testing, using *gene chips*, for Duchenne muscular dystrophy at Emory University to detect mutations in the dystrophin gene. Microarray based testing is extremely sensitive and has a rapid turn around time. "Validation" in this context means that the results of the gene chip method on many samples from Duchenne patients and carriers with different mutations have been compared with the results on the same samples obtained by other established methods. In all cases, the same mutations were found.

The new gene-chip genetic test. In the second part of her presentation, Dr. *Hedge* described this new test which is a 2-step procedure. Step 1 uses a *Comparative Genome Hybridization* (CGH) array of the entire dystrophin genomic sequence of 2.2 million base pairs. In this step, deletions and duplications of exons are identified. Step 2 becomes necessary, when no deletions or duplications are found in step 1.

Step I: These gene chips are printed by the company Roche-NimbleGen Systems Inc. in Madison, Wisconsin.

On a glass slide with dimensions 1 x 3 inch (25 x 76 mm), almost 400,000 DNA oligonucleotides, referred to as the *probe oligos*, are synthesized by a proprietary technique called Maskless Array Synthesizer and attached in a regular pattern, termed an *array*. The oligos are approximately 45 to 60 bases long, and cover, in duplicate, the 2.2 million base pairs of the dystrophin gene. The oligos are synthesized with the desired sequence of the dystrophin gene automatically on the slide. The sample containing the DNA from a patient, is first cut into smaller pieces about approximately the same size as the probe oligos and labelled with a fluorescent dye. When this sample is spread over the array, the DNA from the patient can bind specifically, hybridize, by Watson-Crick base pairing with the complementary probe oligos in the array on the slide. Those probe oligos which have combined with their partners from the patient sample can then be seen and recorded by the fluorescent light produced by the dye on the patient sample after illumination with ultraviolet light. If the patient gene had a deletion, the corresponding probe oligos remain alone, unhybridized, and thus do not produce light. As the sequence and the position of the unhybridized probe oligos are identified, the borders of the deletion, or breakpoints, in the gene can be determined with very high precision. When some exons are duplicated, twice as many probe oligos are paired with their partners, so that the intensity of the fluorescence produced by them is twice as strong.

The results of this method are quantitative, therefore deletions and duplications can be determined not only in the one dystrophin gene of Duchenne boys, but also in either of the two dystrophin genes of women. Therefore, gene-chip testing can help to determine whether a woman is a carrier of Duchenne or not, which is very important for accurate genetic counseling. As this method detects the mutations at the level of the gene itself, it has the advantage of finding the *approximate breakpoints* of the deletions and duplications, i.e., their beginnings and their ends, which are mostly inside the introns. This may be an important finding to further research the reasons for different clinical symptoms of patients with the same mutation. It becomes more and more obvious that introns are not genetic "junk" but contain regulatory sequences which, among other effects, might influence the development of certain clinical signs. If they are disturbed by deletions, it is possible that this could lead to differences in clinical symptoms.

Step 2: If no deletions or duplications are found in step 1, step 2 must be performed, which can identify point mutations, such as small deletions and insertions. For this second part of the analysis, a *Resequencing Array* of 47,000 base pairs is used to look for point mutations (1) in the entire 14,000 base pairs of all dystrophin exons, (2) in the 8 promoters of the dystrophin gene, (3) in the intron sequences of 100 base pair length at the boundary regions of all 79 exons, and (4) in the sequence region around five

known rare mutations inside introns. This resequencing step checks at every nucleotide position in the targeted regions of the dystrophin gene whether the normal base is present or has been replaced by one of the three other possible bases, or whether a base has been deleted or an extra base inserted.

The point mutations, small deletions and insertions, can cause (1) the shift of the reading frame in the mRNA, which leads to Duchenne dystrophy (frameshift mutations), (2) the exchange of one amino acid against another in the dystrophin protein which may or may not cause Duchenne or Becker dystrophy (missense mutations), (3) the appearance of a premature stop codon where the dystrophin synthesis is interrupted (as nonsense mutations), (4) the interference with the splicing mechanism which may lead to exon deletions in the mRNA (splice site mutations), or (5) the damage to a promoter, which may stop the dystrophin synthesis entirely and also lead to Duchenne dystrophy.

Rare exceptions. As with all genetic tests, in some rare cases, this gene-chip test may not find a mutation in a boy whose clinical symptoms indicate Duchenne dystrophy. Mutations may lie outside the regions tested. To address this need, a gene chip with 2.1 million probe oligos in an extremely dense array is being considered. Also in development are gene chips for tests for other neuromuscular diseases, and for the use of dry blood spots from newborn screening programs and of saliva samples. The validation of these methods is in progress.

Advantages. In addition to the advantages of the new test mentioned like high sensitivity in finding almost all mutations in the dystrophin genes of patients and carriers, the main advantage is its rapid turn around time and high sensitivity and accuracy: It takes only about *7 days* for step 1, the detection of the deletions in 60% and of the duplication in 5% of Duchenne boys, and it *takes another 14 days* to find the point mutations in the remaining 35% of Duchenne boys.

The new genetic test is already available. If you wish to have your son or a female relative tested with this test, please contact Emory Genetics Laboratory in Atlanta at www.geneticslab.emory.edu, or tel. 404 778-8500, who can discuss the cost of testing and insurance coverage. For cases where no mutation is detected, a cDNA sequencing using mRNA as starting material is also available. This sequencing test requires a sample from the muscle biopsy. Blood and biopsy samples from outside the US are accepted.

Future of CETT. Within the next few years, the CETT collaboration hopes to arrange a network of clinical laboratories first in the United States, later probably also in other countries, where this fast and reliable test will be available for all Duchenne patients and their female relatives. PPMD with its new DuchenneConnect initiative will provide access to this program and to all information material being prepared for the entire Duchenne community.

DuchenneConnect will encourage collaboration and genetic testing for Duchenne muscular dystrophy.

DuchenneConnect is a groundbreaking information hub that connects the entire Duchenne/Becker community: pa-

tients and families, medical and research professionals, and the pharmaceutical industry to a single source, for the

exchange of data, ideas and guidance, as well as the latest information about research and current treatments.

Kyle Brown of Innolyt Inc. in San Mateo, California, whose aim it is “to connect the non-profit research community”, described the new DuchenneConnect program of PPMD, its relationship to the CETT program, and the need to register all the clinical and genetic information about as many Duchenne and Becker patients as possible. Kyle Brown addressed the families in the meeting in a personal way:

“We want to know the sick children and their families. We want you to enter your information into the Duchenne Connect and CETT programs. We need the clinical and genetic data of your child and wish to know where you live, but we will keep these data apart for preserving your privacy when we let researchers, pharmaceutical companies and other registries have your clinical data with your permission. The list of questions we need to have answered is already long: How was your boy diagnosed? What were the results of his genetic and other tests? How is he walking? Did he have orthopedic surgery because of contractures or scoliosis? What kind of technical aids is he using? Does he need assisted breathing with what kind of equipment? Has he problems with his heart? Are there other members in your family with the same disease?”

This list is not complete, there are more questions on our questionnaires and even more will be added in the near future. This information transfer process will be a continuous question-and-answer affair, because we will have to be in contact with you for a long time.

Through DuchenneConnect, you will be able to see how the disease of your boy develops in comparison with other boys. And we will show you where the best genetic counselors are in the area where you live. And to make our work more and more valuable for you we need your suggestions and ideas which you should bring to us at any time.

Your data in anonymous form, that is, without your name and address or ways to identify you, will be offered to Duchenne scientists everywhere who need them for their research for a therapy. And we will work with other registries in the world, provided they follow the same standards as we do. Also pharmaceutical companies will have access to them, for instance if they need patients with specific mutations for their clinical trials. But if they need to contact you, they will have to ask us whether you had given your permission to share your data. So, you always will be in control of what happens with your quite personal information.

The result of all this will be a large collaboration not only on the national but later even on the international level in which your boy and his entire family will play the most important role.”

Please register with the new internet information center of PPMD to become part of this powerful network: <http://www.duchenneconnect.org>. Currently, English is the only language being used in these internet pages, however, translation into other languages will become available in the near future.

What does “Connect...” mean, the title of the meeting in Philadelphia?

Concluding words by Patricia Furlong. It means that every boy and young man with Duchenne muscular dystrophy *matters* with their families, their doctors and caregivers who make sure that the boys can lead a meaningful life, and with the researchers who are doing everything to find a therapy for them. *Connect* means also that all of them, the entire Duchenne community, must stay together and work together so that Duchenne dystrophy does not cut down any more the usual 70 years or 25,000 days of human life to 20 years or 7,300 days from the day of diagnosis. The present gap of the missing 50 years has to become smaller and smaller until it disappears completely when there is, perhaps not a complete cure, at least an effective therapy in the not too distant future. My two sons died when they were 15 and 17 years old, they did not get the 7,300 days, but that was a decade ago. Now, in the last 10 years, about 1,500 days were added, that is a quarter of a Duchenne life, and the boys have 8,800 days, this is 25 years more or less. So there is progress and it will accelerate with all the therapeutic approaches that are actively pursued in many laboratories and hospitals of the world.

The list of research strategies is long. Many of them have been discussed during this meeting, and you will realize that, with accumulating positive findings and more and more clinical trials underway, we are getting ahead and that there is even real hope that at least one or more of the new techniques, exon skipping and suppression of premature stop codons, have the potential to produce meaningful therapies in the next few years..

Often we worry that our waiting may be fruitless, but this meeting has shown that there is meaningful progress, that not only research advances, but medical care for the boys and young men gets better and better, and, quite importantly, that the public and the governments and their health agencies in many countries realize that Duchenne is a worldwide problem and together we must *end Duchenne*. Substantial amounts of money are becoming available, and our own efforts, which should always be intensified, significantly adds to these funds that make new research programs possible and accelerate longer-term and more mature strategies.

Some of the clinical trials with Duchenne boys, especially the most important ones like those of exon skipping, readthrough of premature stop codons, utrophin upregulation and gene transfer, are using novel therapeutic techniques. Therefore, these trials must be performed very carefully in order to be absolutely safe and effective. More trials will be necessary, therefore, the scientists will need your continued cooperation in spite of the hardship, the travel, and the many, sometimes difficult, test procedures like repeated biopsies, even when no therapeutic benefit is expected. The participation of many more of your boys is essential.

Duchenne dystrophy is not a simple disease. There will probably not be a single therapy which would help all patients. More likely, there will be a combination of different techniques to obtain the most effective way for treating them. And no one will be left out. PTC124 will enter a

phase-III protocol. Exon 51 is the first one to be skipped in the trials, but others will follow, also the difficult, less common mutations. Many trials with more conventional approaches will show positive results which might lead to treatments that will not cure the disease but delay its progression to preserve muscle while waiting until a more effective and more permanent therapy is ready.

And please understand, that many future therapies will be mutation specific. This means that it is important, that the exact mutation of each Duchenne boy must be known in order to decide which kind of therapy would be the correct one for him. Very powerful methods exist with which

the mutations can be precisely analyzed and also those of the mothers and her female relatives which makes genetic advice very reliable.

So, this is our position: All children and young men with Duchenne dystrophy everywhere need and deserve access to accurate and timely diagnosis, genetic testing, state-of-the-art care, the opportunity to participate in clinical trials, and access to promising treatments. In order *to end Duchenne*, we have to connect to each other, and to work together so that the 8,800 days of Duchenne life become 25,000 and more.

This report, written mainly between September and November 2007, is also available in Spanish and German. My earlier reports in English, German, and Spanish can be seen on the internet at www.duchenne-research.com. The 2007 annual meeting of the British *Parent Project Muscular Dystrophy*, PPUK, now called *Action Duchenne*, took place in London from 2 to 4 November 2007. I will update the present report during the coming months with the new research results presented in London. 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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