



Parent Project UK Muscular Dystrophy
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20 Years after finding the Duchenne Gene: A Terrible Disease is being Conquered.

Standing on the Shoulders of Giants This was the title of the 4th International Conference of the Parent Project UK Muscular Dystrophy (PPUK) which took place on 21 and 22 October 2006 in London. Thirty scientists and clinicians for muscular diseases presented and discussed their most recent research results, ongoing and planned clinical trials, up-to-date medical management methods, and registration. I, *Günter Scheuerbrandt*, a biochemist from Germany, was asked by *Nick Catlin*, the president of PPUK, to write this report for you, the boys and their families, who wish to know about each successful step on the way to an effective treatment. As I am not a clinical expert, the report contains 23 summaries of only the scientific presentations, 16 from the scientists present at the meeting, 2 from scientists who were unable to attend, and 5 from other researchers who were not at the meeting but whose work is equally important for the development of Duchenne therapies.

All scientists whose research is summarized, have had the opportunity to see the draft of the summary of their presentation and to correct it if necessary, and all of them have done so, thus there should be very few mistakes left. Another consequence is that even results are reported which were obtained between the conference and the writing of this report in January 2007.

After the annual meeting of the American Parent Project Muscular Dystrophy in Cincinnati/Ohio from 13 to 16 July 2006, I have written a similar report which can be seen on the internet at www.duchenne-research.com. The two reports belong together and both are not scientific publications, they are written for you, the boys and your parents, in a language you should be able to understand.

In general, I am using the names of the scientists without their titles, most are professors and all have either a PhD or MD title or both. And almost all are heads of laboratories, that means they have colleagues and postdocs and students working as a team on the projects reported here, but it is impossible to mention all their names.

About 140 years after the description by *Guillaume Duchenne de Boulogne* and 20 years after *Louis Kunkel* isolated its gene, the dystrophin gene, this terrible disease, Duchenne muscular dystrophy, is slowly loosening its grip, this is obvious after the presentations of so many new research results at these two meetings. Duchenne muscular dystrophy is being conquered step by step by so many dedicated people working for us in many countries. The two reports are showing why this is so.

Introduction by Nick Catlin: Standing on the Shoulders of Giants

In 1675, Isaac Newton wrote to Robert Hooke that in order to make great advances in Science we must *Stand on the Shoulders of Giants*. Of course he was being rather too humble and perhaps making a point to Hooke that he should take more time studying the likes of Copernicus, Kepler and Galileo than frequenting the London Coffee houses. During this conference we are very privileged to have some of the scientists speaking who have made vital discoveries that have opened the possibilities for new gene therapies for Duchenne Muscular Dystrophy. For more than 100 years since the first description of the disease by Duchenne, we had little knowledge of the causes of DMD. In 1986, this is the 20th anniversary, the dystrophin gene structure was discovered by *Luis Kunkel*, *Anthony Monaco*, *Kay Davies*, *Eric Hoffman* and others. Without their work we would have little hope of finding a cure for Duchenne today.

There was a great surge of optimism that initially followed the discovery of the gene structure. But in 20 years we have lost another generation of boys to this terrible muscle wasting disease. A deep general pessimism had set in by the time my son Saul was diagnosed in 2000. Charities and scientists alike dared not to speak of cures or treatments to parents and even the use of steroids was still not

commonplace. Many scientists had left the field of Duchenne research as funding dried up and neuromuscular diseases seemed to be forgotten in the global fight against cancer, AIDS and other diseases that affect larger groups of the world population. However dedicated groups of researchers, scientists and clinicians and parents have battled on trying to overcome a devastating disease that leads to total paralysis and early death. We also now know that many young boys are also affected by related learning and behaviour problems.

For me and PPUK the most significant turning point came when we lobbied for £ 1.6 million of Department of Health funding in 2003 for the UK MDEX exon skipping consortium. This was a watershed in terms of our government at last waking up to the needs of Duchenne families and providing significant funding for a major gene therapy research project. The MDEX consortium of clinicians and scientists have broken new ground in collaborative research and we have great hopes for the first gene therapy for DMD. Since then other clinical trials have mushroomed and we now have biotech companies like VASTox in the UK, Prosensa in Holland, PTC and AVI from the USA, and Santhera from Switzerland not only adding Duchenne projects to their portfolios but seeing these as lead-

ing project developments.

This trend of new research projects and further clinical trials seems set to continue into 2007. MYOAMP has been established from a large European grant to accelerate promising advances in muscle stem cell therapy. Treat NMD has secured a € 10 million budget to put together new networks of scientists and clinicians to promote better clinical practice and accelerate clinical trials. PPUK through the wonderful efforts of many UK Duchenne families has now funded six key projects for some £ 300,000 in partnership with our international Parent Project groups in France and Monaco.

Twenty-year anniversary: Finding the dystrophin gene and its protein.

In 1980, *Louis Kunkel* started work as a postdoctorate fellow at the laboratory of *Samuel A. Latt* at the Children's Hospital of Harvard University in Boston with the intention to finally find the cause of Duchenne muscular dystrophy, 118 years after Dr. *Duchenne* in Paris described correctly this most frequent hereditary disease of childhood. He asked the American Muscular Dystrophy Association MDA to fund this project, but they did not believe that he could really find the Duchenne gene, so he told them how he planned to do it: (1) Mapping the gene, that is, finding where exactly it is on the X chromosome; (2) checking whether and how it is mutated, damaged, in Duchenne patients; (3) identifying the coding sequences, those many separated strings of genetic letters, the exons, that contain the information for making a protein; (4) putting these exons together, that is, making the so-called cDNA consisting of these active parts of the gene; (5) predicting the amino acid sequence of the protein with the help of the genetic code; and (6) finally isolating the protein. That convinced the MDA, and Louis got the money.

At this meeting, *Louis Kunkel* described these steps, which led him to his goal. At that time, a quarter century ago, it was much more difficult and time-consuming than today to isolate a gene and then to predict and find its protein. Here are, very abbreviated, the steps taken to find the gene:

It was known that the Duchenne gene must be on the X-chromosome, because – with very rare exceptions – only boys get the disease and their mothers are often the genetic carriers. *Kay Davies* and *Bob Williamson* separated the entire X chromosome from the others using new techniques. Markers were found, short genetic sequences or tags, which allowed to pinpoint the position of the gene on the chromosome in relation to the many other genes. Already at this stage it was realized, that there was only one gene for Duchenne and Becker dystrophy. And with *Gertjan van Ommen*, methods for early and prenatal diagnoses were developed which even then made genetic counseling much more precise.

Then some unusual patients helped to approach the Duchenne gene further. There was a woman who had Duchenne symptoms. *Ronald Worton* found that a small piece of the short arm of her X chromosome was attached to a shortened chromosome 21, and the missing piece of chromosome 21 was now located where the X chromosome had lost that part of its structure. Thus, a translocation, an exchange, of chromosome material had happened in all her cells. The scientists could see these altered chro-

But this is not the time to sit back and wait. Our families must understand the urgent need to redouble our campaigns and fundraising efforts if we are to save this generation of boys from this devastating disease. We have to take as our inspiration and hope those scientists that have paved the way for the progress being made today. We also have to stand on the shoulders of giants like Pat Furlong in the USA who has refused to give up the fight to cure DMD despite losing her own two sons. It was Christopher Furlong who told her before he died "If you don't fight for a cure Mum - no-one else will"

mosomes under the microscope, thus they could localize the breakpoint. And because the female patient had Duchenne symptoms, this breakpoint must have been inside the Duchenne gene and thus disrupted and inactivated it.

Then a child appeared who had Duchenne dystrophy and also three other diseases with the same mode of heredity. So with *Uta Francke*, it could be shown that all three genes were missing, there was a very large deletion in the X chromosome that could be seen. Other patients turned up with smaller deletions also close to the place where the Duchenne gene was supposed to be. Then many pieces of normal chromosome material which represented the deleted regions were isolated, and among them was one, called PERT87, which actually detected deletions in 5% of Duchenne boys. Many more researchers started to work with this short chromosomal sequence which through its very specific DNA base pairing attached itself to the complementary sequence inside the Duchenne gene and nowhere else. But it does that only if the target sequence was there; if it was not there, deleted, the PERT87 could not find it and thus the scientists could distinguish patients with and without a deletion at that site.

Louis Kunkel with his growing team collected all the data from his colleagues and finally could analyze genetic details of 1,346 Duchenne and Becker patients, still the largest study of this kind. Because 8.7% of the patients had a deletion at the PERT87 site, the Duchenne gene had to be there. Because this gene is very important not only for humans but also for all other animals with muscles, similar experiments with PERT87 were done on muscles from mice, chicken, and monkeys, and indeed, this gene was conserved during evolution. Finally, clones, i.e., copies were made from the area around the PERT87 site until all coding sequences, the 79 exons of the gene, were identified.

So, six years after Dr. Kunkel started his work, in 1986, he and his colleagues really had done it, they had the gene with, as is now known, exactly 2,220,223 nucleotides or genetic letters. It is by far the largest of the about 22,000 human genes, representing 0.1% of the entire genome, and it is still not known why such a large gene is needed.

From the sequence of the cDNA and with the help of the genetic code, *Michel Koenig* and others in Kunkel's laboratory were able to predict the structure of the protein whose production was guided by this gene. It had to be a rod-shaped protein chain of 3,685 amino acids. But where was it located? Together with *Eric Hoffman* and *Kevin Campbell*, two large coding sequences from several exons

of the cDNA were isolated and then transferred into coli bacteria which then produced, expressed, large quantities of two proteins which actually were short stretches of the Duchenne protein. These shortened proteins were injected into rabbits, which treated them like vaccines and made antibodies against them. After attaching fluorescent tags to these antibodies, the research teams of *Ronald Worton* then in Canada and *Hideo Sugita* in Japan, and Kunkel's group also, could show that the protein was located at the underside of the muscle fiber membranes. It revealed its presence there by producing fluorescent bluish light around the rim of vertically cut muscle fibers under the microscope, a technique which still is used to prove the presence of this Duchenne protein or its absence in muscle tissue.

So, one year after the gene was found, its protein was also known, which the researchers named *dystrophin*. And the gene was no longer the unknown Duchenne gene but the now well-known *dystrophin gene*.

These discoveries led to the development of molecular diagnostic methods. *Jeffrey Chamberlain* and his group used the new polymerase chain reaction, PCR, for detecting deletions in the dystrophin gene and found that about 65% of Duchenne patients have such deletions. *Kevin Flanigan* and again Kunkel's group started working on high-speed sequencing methods to detect point and other more rare mutations in the gene.

Then *Eric Hoffman* realized that, while Duchenne patients have no or very little dystrophin in their muscles,

Becker patients have altered dystrophins, and *Anthony Monaco*, while trying to explain this finding, came up with the reading frame-hypothesis in 1988, which, with some exceptions, is now the proven basis for the *exon-skipping* technology. In fact, this now very promising method to restore the reading frame was discussed and proposed as a possible therapeutic approach among the researchers at that time already, but nobody knew that early how to eliminate exons from the messenger RNA.

One other important consequence of *Louis Kunkel's* and his colleagues's work was the detection of the genes for the many limb-girdle muscular dystrophies, LGMDs, because when they pulled the dystrophin out of the muscles with antibodies, a rather large number of other proteins were taken out, too. As *Kevin Campbell* in Iowa City and *Eijiro Ozawa* in Tokyo showed, they belonged to the dystrophin associated protein complex DAPC which anchors the dystrophin to the outside of the muscle cell membrane. It is now known that mutations in the genes of these proteins cause the different forms of LGMD.

Now, 26 years after setting out to find the gene, *Louis Kunkel* is still working on muscular dystrophy at the same children's hospital in Boston, concentrating on cell transfer techniques with myoblasts and other muscle stem cells which show promise of becoming therapeutic methods in addition to the other techniques. He finished his presentation with the statement: "We are in a stage now where it is not hopeless anymore for a child now born and diagnosed with Duchenne".

Why do we need clinical trials?

In her first presentation, *Kate Bushby* of the University of Newcastle upon Tyne explained that clinical trials are very much needed for the development of an effective drug and that these trials can take much time. But Duchenne boys do not have many years to wait until such trials are performed and finally a drug is developed. So they and their parents should understand that the scientists working for an effective therapy do appreciate their situation and are working with the regulators to be sure that effective treatments could come to trial as quickly as possible. In the interest of the families and their sick children, the scientists, when they have an idea, a hypothesis, how a therapy might work, have to work step by step, even before clinical trials can be started, in order to be certain that each research step gives valid answers on which more research can be based, first with small experimental animals, the mdx mouse for example, then with larger animals, the dystrophic dog or monkeys, until finally the technique developed through these steps can be tested in living patients, the Duchenne boys. Any mistakes, sometimes caused by dangerous shortcuts, cannot be tolerated because they would delay the research progress by many years.

Duchenne muscular dystrophy is a complex disorder and effective treatments in the long run will probably have to act on the genetic machinery that makes dystrophin in healthy but not in Duchenne 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 many pounds of muscles of a boy, even the inside ones like those of the lungs and the heart. Therefore, the demands for the safety and the efficacy of such a Duchenne drug are very severe.

To understand what clinical trials are, *Kate Bushby* mentioned four rules:

Rule 1: Trials are about testing a hypothesis, an idea that makes sense. They are there to answer a question for which an answer is needed – for example "does this drug work to cure DMD?" – and they do not necessarily give the expected answer. Such a hypothesis should be based on good and reliable preliminary data.

Rule 2: Being a participant in a trial should not be a substitute for receiving the best available care. One must not drop everything one knows, after all, a trial might not work.

Rule 3: The design of a trial is dictated by the nature of the hypothesis to be answered. It might be a pilot, open label, blind, or double-blind placebo-controlled trial. The number of participating patients depends on what degree of data reliability is needed to answer the question, more participants make the results more precise and more reliable. 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 actually experience any improvement as these kinds of trials are set up to answer safety and limited efficacy questions only: (1) Phase I to test for toxicity, (2) phase II to test for efficacy, dosage and safety, and (3) phase III to confirm a clinically relevant positive effect and determine the optimal dose. Trials with children who have a progressive disease like Duchenne are very challenging, they have to be designed very carefully because young children with DMD grow and become better even without a drug.

Rule 4: 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. The ethical considerations are more than only issues of consent by parents and the patients themselves, but also 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 following and the final regulatory approvals. The extensive paperwork, the long delays, and the rather large cost of clinical trials make sure that everything is being done correctly.

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 regulation for tax savings are important. (2) Because Duchenne dystrophy is quite rare, patients with specific mutations in their dystrophin gene will be scarce, often caused by the often missing full molecular diagnosis. So parents should insist that the exact mutation in the dystrophin gene of their sick son is determined as soon as possible. (3) The establishment of registries with the full diagnostic data of as many patients as possible from all over the world would be a tremendous advantage for the design of future trials, and families

should be encouraged to find out about the existence of such registries and enrol their child. (4) A real trial culture among in the Duchenne community of families, doctors, and scientists with international cooperation must be developed in as many countries as possible. There is a new international collaborative effort run by *Kate Bushby* and her colleague *Volker Straub* – TREAT-NMD: www.treat-nmd.eu – which hopefully will accelerate the progress of promising molecules into trials and treatments.

There are more negative than positive clinical trials, so no patient should stop or neglect the best possible medical care that is already available. For the same reason, it does not make sense to travel large distances to take part in a trial when there is still no idea if it is going to give a positive result. Consent should always be given voluntarily after full explanation of all positive and negative details. Discussions with the scientists and doctors should always be possible, and the parents should be allowed to withdraw their sick son from a trial at any time without having to defend their decision.

Kate Bushby said finally: 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.

Exon skipping.

How exon skipping works: Exon skipping is one of the potential therapeutic techniques that is already being tested clinically on Duchenne patients. In his introductory presentation, *Steve Wilton* of the University of Western Australia in Perth, described this technique in detail. The readers of this report who are not familiar with the biochemistry of how genes make proteins, of the structure and function of the dystrophin gene and its protein dystrophin, and how mutations cause Duchenne dystrophy, should please read first the introductory chapters of the report on the Parent-Project meeting in Cincinnati/Ohio in July 2006. This report is available in English, German, and Spanish on the internet at www.duchenne-research.com.

The *exon skipping* technique tries to change a Duchenne mutation into a Becker mutation, so that the severity is reduced. If a mutation disturbs the reading frame and thus causes Duchenne dystrophy, the reading frame can be *restored* by artificially removing from the messenger RNA one or more exons directly in front or after the deletion, the duplication, or the exon which contains a point mutation. In the latter case, either removal of the mutated exon alone may by-pass the defect, or it may be necessary to remove one or more neighboring exons to maintain the reading frame.

Exons can be eliminated from the mRNA with *anti-sense oligoribonucleotides*, AONs. They are short RNA-like single-stranded compounds consisting of 20 to 30 nucleotides whose sequences are constructed in such a way that they attach themselves by Watson-Crick base pairing only to the complementary sequence inside the exon to be removed or to its border regions. *Antisense* means that their base sequence is complementary to the target sequence in the pre-mRNA. 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*.

Splicing the exons of the pre-mRNA to the mRNA is a very complicated and precise procedure mediated by a complex of many proteins that recognize the borders between the exons and the introns. The AONs have to have a sufficient long nucleotide sequence so that they inhibit the splicing of only those target exons which are necessary to restore the reading frame of the defective dystrophin mRNA. At present 231,677 exons in the about 23,000 human genes are known. The exon skipping process, therefore, has to be extremely specific and precise. If the AONs would cause skipping of exons in other genes, dangerous side effects would be the consequence.

Exon skipping does not alter the gene itself with its mutation, but its mRNA no longer contains the information of the skipped exon or exons, and not of the deleted exons either. This therapy affects how the defective gene is read and processed. 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 central rod domains, the shorter protein can often still perform its stabilizing role of the muscle cell membrane. The result would be the change of the severe Duchenne symptoms into the much milder symptoms of Becker muscular dystrophy.

Oligoribonucleotides are short pieces of RNA - oligo means few. *Nucleotides* are the building blocks of nucleic acids. They consist of three molecular units: one ribose, one base, and one phosphate. So there are four different *ribonucleotides*. The two kinds of AONs, which are mostly used for exon skipping, are protected oligoribonucleotides so that they are not or only slowly destroyed in the muscle cells by nucleases, enzymes, that destroy nucleic acids.

The Dutch scientists are using *2'-O-methyl-phospho-*

thioates, also called *methyl thioates* or *2O-methyls*. They have a methyl group, a carbon with three hydrogen atoms, on the oxygen of the second carbon of the ribose units, and a sulfur atom instead of one of the oxygen atoms of the phosphate groups. The *morpholinos*, which the Australian researchers have found most promising, and the British will use in their planned trial, have one of the phosphate oxygens replaced by a dimethyl amide group, a nitrogen carrying two methyl groups, and the entire ribose units are replaced by morpholino rings, six-membered rings, each consisting of four carbon atoms, one oxygen and one nitrogen atom with hydrogen atoms attached to the carbons.

In Dr. Wilton's laboratory, morpholino AONs are being developed, tested, and optimized, so that all dystrophin exons can now be skipped, one alone or several at the same time, in cell cultures of normal and dystrophic mouse, dog, and human muscles. Some exons are skipped more easily than others. Exons that are difficult to remove from the mRNA need higher concentrations of the AONs, but work continues to optimize their structures. Morpholinos AONs are probably quite safe in humans, because they have been tested already in adults, not in children, as antibiotics to destroy viruses.

Exon skipping will *not* be a cure for *Duchenne dystrophy*, it should reduce the severity of its symptoms thus converting it into Becker dystrophy with a better prognosis. It will probably benefit up to 80% of all Duchenne patients. The first clinical studies, one using 2O-methyls and the other morpholinos as described below, will target exon 51 locally in a single muscle to establish the *proof of principle*. Systemic trials with injections of the AONs into the blood circulation will follow soon. The researchers will continue to work with and to test clinically both kinds of AONs, because it might happen that negative results in future clinical trials with one AON type will make it advisable not to use it in the long-term studies. It is even conceivable that combinations of both AON types could be used in the future.

Many details about exon skipping were discussed in an interview with *Steve Wilton* which is part of the report on the Cincinnati meeting. That report contains on its last page also a detailed example of the skipping of exon 46 to restore the reading frame after the deletion of exon 45.

Exon skipping in the heart: At the beginning of his presentation, *Dominic Wells* of Imperial College London summarized his exon skipping experiments with mdx mice. He used a *morpholino antisense oligoribonucleotide*, AON, that contained 25 ribonucleotide units whose sequence was directed against the last 7 bases of exon 23 and the first 18 bases of the following intron 23 of the mouse dystrophin pre-mRNA. This AON attaches itself exclusively to the complementary sequence at the border region of exon 23/ intron 23. This prevents the splicing machinery inserting exon 23 between the exons 22 and 24 of the mRNA. *It skips exon 23*. And this is intended, because in the mdx mouse, this exon has a point mutation at position 3,185 which changed the codon CAA for the amino acid glutamine to the premature stop codon TAA. Therefore, the production of dystrophin terminates at this stop sign and the animal does not have any or very little dystrophin in its muscles. The elimination of the entire exon 23 removes this stop signal, and because both borders of exon 23 are between entire amino acid codons, the skipping of

this exon does not disrupt the reading frame. The ribosomes, the "protein factories" then make a shorter than normal dystrophin, 72 amino acids are missing, meaning that the new dystrophin is about 2% shorter than normal. After only one local injection of the specific AON into a single skeletal muscle, the muscle recovered its function significantly and expressed, produced, dystrophin for at least 10 weeks. Previously published work by *Terry Partridge's* group has shown that in a systemic application, a similar normalization of the structure and function of many skeletal muscles was obtained after 7 weekly intravenous injections of 100 mg AON/kg bodyweight in 6-week old mdx mice. *However, it was not possible to get the morpholino AONs into the heart muscles of the mice*. This was a severe disadvantage of the morpholino compared with the 2O-methyl AONs which at high doses do enter the heart muscles and cause exon skipping.

Julia Alter in *Dominic Wells's* laboratory was able to change this situation by applying ultrasound with commercial diagnostic ultrasound equipment focussed directly to the heart of the mice. The ultrasound causes temporary pores in the cell membranes of the cardiac muscles. This allows the morpholino AON molecules to pass into the interior of the cells. When the ultrasound treatment is discontinued, the pores in the membranes disappear again. And this effect was significantly enhanced by applying at the same time the contrast agent Optison which is gas-filled albumin bubbles of about 2 micrometer diameter.

This very simple-to-apply technique increased substantially the number of dystrophin-positive fibers in the heart. It did not interfere with the AON treatment of the skeletal muscles, it has even been shown to increase the exon skipping effect in the gastrocnemius, the calf muscle, and no side effects have been detected.

This simple technology uses an evaluated and often-employed diagnostic procedure which one should consider to apply in the systemic clinical exon skipping trials not only with morpholinos but also with 2O-methyl AONs where it should also enhance their uptake into all types of muscles.

Exon skipping: Preparation of a clinical trial in the United Kingdom: In the United Kingdom, the *MDEX Consortium* was established in January 2005 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*, all of them are active in Duchenne research. The Department of Health, the Medical Research Council, the Parent Project UK, and the British muscular dystrophy association, Muscular Dystrophy Campaign, are also involved. In addition, the MDEX Scientific Advisory Board (SAB) consisting of *Kay Davies, Serge Braun, Ian Eperon, David Hilton-Jones, Chris Mathew, and Stephen Meech* as well as non-voting representatives from several associations are meeting every 4 to 6 months to oversee and validate the consortium's work.

At the meeting, the chairman of the MDEX Consortium, *Francesco Muntoni* of the Imperial College in London, reported at the on the state of the preparation for the upcoming first trial and the plans for the future. For this trial, a number of decisions have already been made: The exon to be skipped will be *exon 51*, because the largest

group of Duchenne mutations like the deletion of exons 45-50, 47-50, 48-50, 49-50, 50, 52, 52-63, about 19% of all Duchenne deletions, could be treated by skipping just this one exon 51. Six different 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 that after injection into mdx mice, these types of AONs were still present after 14 weeks, which shows that morpholino AONs are sufficiently stable for the long-term treatment that will be necessary for a life-long therapy of Duchenne boys.

Three groups of two Duchenne boys each, 12 to 18 years old, will participate in this first trial. After all three relevant authorities, the Gene Therapy Advisory Committee (GTAC), the Medical and Healthcare Product Regulatory Agency (MHRA), and a local committee have given their approval, the first boy will be recruited and will receive his injections in March 2007. Three different dosages: 0.09, 0.297, and 0.9 mg AON in 0.9 ml solution will be used for each group, delivered into a volume of one cubic centimeter of muscle with nine injections directly into one of the two *extensor digitorum brevis* (EDB) muscles on the outside of the foot that is needed only to wriggle the toes. Humans do not really need it and 0.8% of the general population do not even have it. So it can be removed without serious consequences if some unacceptable side effects should occur. Extensive clinical checks including biopsies will be done before and 30 days after the injections to assess the results of the treatment. The highest dose will only be used if the lower doses are not sufficient. And the trial will not continue if clear results, positive or negative ones, are obtained in the first two patient groups.

No preliminary results on this first trial will be released, unless there are negative results which will suggest that the trial has to be stopped on safety grounds. The final results of the entire study will be put together for communication as soon as they are analyzed and approved by the MDEX Scientific Advisory Board.

This first trial is only a very small step, it just will 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 with the different dosages used dystrophin would appear in more than 10% of the muscle fibers. This would allow to get reliable results and also to estimate the total amount of AONs needed to treat all the muscles of a boy in a future systemic treatment.

The boys participating in this first trial with morpholinos injected locally will not get any therapeutic benefit. But all the results of this trial will be needed for a real treatment, for a systemic application of the potential Duchenne drugs into the blood circulation of a boy so that all his muscles can be reached. If the first local trial is successful, this second and more important trial with systemic intravenous injections will start in the second half of 2007, its results should be available in 2008.

As far as the commercialization is concerned, this will depend on the results of the trials. If the British or the Dutch approaches do not work systemically, it is unlikely that the commercial development of AON drugs will be-

gin. However, if they show that the systemic application works with sufficient efficacy in Duchenne boys, then it is hoped that shortly afterwards, there could be bulk production of AONs targeted at many exons for extended clinical protocol studies.

Exon skipping: The first clinical trial in the Netherlands. At the meeting in Cincinnati, Gerard Platenburg, president of the biotechnology company Prosenza B.V. in Leiden, the Netherlands, and, at the meeting in London, Judith van Deutekom of Leiden University Medical Center reported on *the first in-human trial with the exon skipping technique*.

The aim of this trial is to prove that exon skipping is safe and works effectively in Duchenne patients. It is a "local study" in a small area of a single muscle, the *tibialis anterior* muscle of the shin, which is being treated with an antisense-oligoribonucleotide, AON, against exon 51. The trial will only provide a *proof of principle* and although shortened dystrophin is generated, no therapeutic benefit of the treated boys is expected, as treatment is applied locally and only once.

Exon 51 was selected as the first skipping target because successful skipping of this single exon would allow restoration of the protein reading frame for almost 25% of all Duchenne boys with deletions. For this trial, four 8-to-16-year old Dutch Duchenne boys, have been selected. Each boy has a different deletion, namely of the exons 50, 52, 48-50, and 49-50 respectively. The study is open, meaning everybody concerned knows that all four boys are receiving a potential Duchenne drug.

Because exon skipping is an unprecedented new medical procedure, intensive clinical and molecular genetic tests and a skin biopsy were performed on each boy before they were allowed to take part in this trial. From the biopsy material, cell cultures were prepared in which the particular deletion was determined in the DNA and also in the messenger RNA and – in order to be completely certain – the expected base sequences of the border regions around the deleted exons were confirmed in detail. In addition, the entire dystrophin gene was screened to make sure that there were no unexpected irregularities. This special care had to be taken, because this first human application of exon skipping will influence decisively the further development of this potential Duchenne therapy.

For this trial, the Dutch researchers have selected the 2'-O-methyl-phosphothioate version of the anti-51 AON – also called a 2O-methyl – because they have extensive experience with these 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.

In this currently ongoing trial, each boy received one single injection under local anaesthesia into a small area of the *tibialis anterior* muscle of a solution containing 0.8 mg of the anti-51 2O-methyl. Four weeks after the injection, a muscle biopsy has been or will be taken and the muscle tissue checked for the presence of the shortened, skipped, dystrophin protein and its messenger RNA.

No serious adverse reactions were observed in all four treated patients. Two biopsies had already been performed at the end of October and the first results of the RNA and dystrophin analyses in the obtained muscle tissue were quite promising! Although Dr. van Deutekom was not able

to provide more details at this time, she could say that the procedure seems to work rather nicely and that the study can be continued and completed as planned.

The Dutch researchers are now preparing the next clinical trial during which they will try to skip exon 51 by *systemic application* of the appropriate 2O-methyl AONs into the blood circulation so that the potential drug can reach all muscles including those of the lung and the heart.

These studies will be short-term trials that will probably be followed by long-term studies, which could possibly already could slow down the boys' Duchenne symptoms significantly. How much the muscle function will be improved will depend on the Becker-like proteins obtained after skipping. Some will work better than others.

At the end of her presentation, Dr. *van Deutekom* asked the parents to be careful and not to jump to premature conclusions. "*We are not there yet*", she said. Even if exon skipping works in one patient, it does not mean it will work in others. All the data have to be available before it can be said it really works after a local application. But the treated area is very small, therefore no improvement of muscle strength is expected. That was not the aim of this first study, but only the proof that exon skipping works in principle and is safe. However, local intramuscular injections will not be the way to treat patients. Therefore, before starting further trials, more data and more animal studies are needed, to find – among other things – the best AON dosage for a systemic full-body treatment.

The answer to the question "When will it be on the market?" was, that it will take at least five more years to have the AON for exon-51 skipping ready. If this works well, the development of other AONs will follow soon after. In addition to the 2O-methyl AON for skipping exon 51, the company Prosenza has already designed and pro-

duced in sufficiently large quantities four other 2O-methyls. These AONs would allow treatment of over 50% of all patients with deletions. Drug development needs a lot of testing, takes a lot of time and money. Without Prosenza, things may have gone much slower. Even with their engagement it took two years to prepare and to begin the present clinical study.

To the question of how patients could participate in the next studies, the answer was that if the clinical and genetic data of the boys are in the Dutch data bases, the parents will be called if their son fulfils all the inclusion criteria and is needed for the trials.

Judith van Deutekom finally thanked above all the Parent Project organizations in the different countries but also other funding agencies for their financial support and asked the boys and their parents to have patience and confidence because "We will get there!"

Systemic exon skipping in the dog: *Terry Partridge* of the Children's National Medical Center in Washington, who was not present in London, sent the following message on 23 January 2007:

In the Japanese dystrophic dog colony, the mutation has been bred onto a beagle background. One 5-month old dog was given intravenous infusions of the morpholino combination designed to skip the mutation. Two weeks after the last injection, he had significant amounts of dystrophin in many of his muscles, the muscle pathology looked better and he had maintained his running speed. His two non-treated siblings had no significant amounts of dystrophin and had deteriorated in all measures used. No toxic effects were noted in the treated dog. We are just about to start further experiments on two dogs using higher doses and delivering the original dose over a longer time period.

Transfer of the dystrophin gene.

Viral Vectors and muscle stem cells: *Jennifer Morgan* of the Dubowitz Neuromuscular Unit of the Imperial College in London started her presentation with a description of the different vectors that are used for the transfer of the entire coding sequence of the dystrophin gene or parts of it into muscle cells of Duchenne boys. The word *vector* is used for the molecular transporters of the gene sequences. They are mostly viruses which normally infect living cells and bring their own genes with them which then instruct the cells to multiply them. Scientists have learnt to grow non-pathogenic viruses – that do not cause severe diseases – in the laboratory after they have taken out all their multiplication genes but leaving what they need to get into their target cells. This leaves room for foreign genetic material, which can be introduced into their almost empty protein shell. These modified viruses can still infect their target cells, but they cannot be multiplied in them anymore. The transported foreign genetic material is deposited in the cell nucleus, either inside or outside the chromosomes. Under the right circumstances, this foreign material, this foreign gene, can become active and take over the function of a damaged gene that cannot work properly any more. Modified viruses carrying all or only some of the coding sequences of the dystrophin gene, can thus cause the dystrophin protein to be made again. Thus, viruses can provide a genetic therapy, they can be a *genetic drug*.

There are a number of different kinds of viruses which have been used as vectors in gene therapy experiments. The one type, with which the Duchenne researchers mostly work to restore the dystrophin protein in muscle cells, are the *adeno-associated viruses*. They are small viruses that have a single-stranded DNA molecule as their normal genetic material, their normal genome. They can enter the post-mitotic skeletal muscle cells which make up 40 to 45% of the body weight of a human being. Post-mitotic means that these cells do not undergo mitosis, they are not dividing any more. Because these viruses are so small, they can only accept foreign genetic material that is not longer than about 5,000 nucleotides. Thus, they can only transport about one third of the dystrophin cDNA, one third of the joined exons of the gene. The vector construction now used in the first dystrophin-gene clinical gene transfer trial performed in Columbus/Ohio contains a micro dystrophin cDNA which makes a shortened dystrophin protein consisting of only 2,539 amino acids, that is 31.1% of the 3,685 amino acids of the normal dystrophin. Because of this shorter-than-normal dystrophin, this type of gene therapy will not cure Duchenne dystrophy completely, but, like exon skipping, will only reduce the severity of its symptoms to those of the much slower progressing Becker muscular dystrophy.

The adeno-associated viruses deliver their cargo, the

therapeutic gene material, into a chromosome at random, and it cannot be predicted or controlled where this happens. They may insert the transferred gene between two other genes, inside an intron or an exon, and thus may activate, deactivate or destroy another gene. This can cause another disease or even cancer when a proto-oncogene is activated.

For this and other reasons, Dr. Morgan and her research team have started to work on another strategy: the *combination of stem cells with gene therapy*. The satellite cells of muscle fibers are stem cells that can give rise to complete new muscle cells in damaged muscle tissue. Some of them divide asymmetrically, as stem cells often do, and thus create more satellite cells which then place themselves at the outside of the muscle cell membrane from where they are able to regenerate the muscle cell if it is injured. In fact, satellite cells from a healthy donor, a person with normal dystrophin genes, have shown to cause the production of new and normal dystrophin after they were injected into the degenerating muscles of a Duchenne patient. Other types of stem cell have also been shown to regenerate skeletal muscle, e.g. synovial stem cells and mesangio blasts. We will find the stem cell that gives the best skeletal muscle regeneration and use this cell type in future experiments.

Lentiviruses are a type of retroviruses with RNA as their genetic material which integrate into the host cell's DNA and create a double-stranded DNA copy inside the infected cells. These viruses can infect dividing and non-dividing cells, thus also satellite cells of muscle fibers. Dr. Morgan's research team used such lentiviruses to transfer the genes of a modified U7 splicing factor with an antisense sequence for skipping exon 51 into satellite cells. This is a similar technique as developed by the French scientists around *Luis García*, who are using adeno-associated viruses as vectors. Dr. García provided Dr. Morgan with the lentiviral U7 construct. In preliminary experiments with this lentivirus technique, skipping of exon 51 was obtained in cultures of human muscle cells after a few hours. But these are very preliminary results of experiments which have to be repeated and developed further. More news about this additional approach for a Duchenne therapy are expected soon.

First clinical trial of dystrophin gene transfer in the USA: *Xiao Xiao* of the University of North Carolina in Chapel Hill, could not attend the meeting in London, but he sent the following report on the current status of AAV mini-dystrophin gene therapy for Duchenne dystrophy:

Gene therapy is one of the numerous strategies being vigorously developed for the treatment of Duchenne dystrophy. Among the gene therapy approaches, adeno-associated virus (AAV)-mediated gene delivery of a functional mini- or micro-dystrophin gene has some apparent advantages. It is highly effective in muscle and heart and able to cover almost the entire spectrum of the Duchenne patients. Preclinical proof-of-principle studies in small and large animal model systems have supported the development of mini-dystrophin gene therapy for clinical trials.

Through collaboration between the University of North Carolina, the Ohio State University and Asklepios Biopharmaceutical company, and supported by the American Muscular Dystrophy Association MDA, a phase-Ia gene therapy clinical trial has been initiated in March of 2006. It

was approved by the Federal Drug Agency FDA following safety and toxicology testing of the AAV mini-dystrophin vector in laboratory animals.

It is a randomized, double-blinded trial with six patients and two different doses involving local intramuscular injection of the gene vector. The major objectives are. (1) to collect safety data including the patient's history of symptoms, febrile reactions, swelling or erythema – skin inflammation – at the injection site, measurement of changes in serum chemistries and hematology, urinalysis, pulmonary function testing, and isometric muscle strength testing; (2) to determine the dose required to achieve the production – expression – of the mini-dystrophin in muscle; and (3) to monitor potential immunological responses to the mini-dystrophin as well as to the delivery vector itself.

So far, all three patients in the low-dose group have been injected and muscle biopsies collected. No gene therapy-related adverse events were observed. Two of the three patients in the high-dose cohort have also been injected. No gene therapy related adverse effects have been observed in them either, suggesting that the procedure is well tolerated. Due to the nature of double-blinded design, the muscle biopsy data will not be analysed until all six patients are injected and muscle samples collected. Results from preliminary analysis of these patients are expected in late summer of 2007.

Our next goal is to develop regional gene delivery methods – similar to the one used in the French plasmid trial – to treat large groups of muscles and eventually the entire body. We have investigated this retro-grade, intravenous perfusion method in the limbs of normal and dystrophic dogs with both reporter genes and the dog mini-dystrophin gene. Encouraging gene transfer and expression results have been obtained. In addition, we have also initiated the investigation of this method in monkeys using a reporter gene. Our preliminary data revealed widespread gene expression in various muscle groups in the hind legs we tested. However, extensive, short- and long-term studies are required to optimize the gene delivery system and to establish safety and efficacy in large animals before the phase-Ib clinical trial of regional gene delivery can take place.

At the Cincinnati meeting, *Scott McPhee* of Asklepios reported that the vector used in this trial is a modified AAV of serotype 2, called BNP2.5, containing a mini-dystrophin gene which does not contain parts of exon 17, all exons from 18 to 59 and from 70 to 79 inclusive. That means that the expected Becker dystrophin will be about one third as long as the normal protein because it lacks the rod regions R3 to R21 and the C terminal end.

Exon Skipping with U7 gene transfer: *Luis García* of the Génethon Institute in Evry near Paris presented the results obtained in his laboratory on an approach to *combine exon skipping with gene therapy*. The French researchers are attempting to instruct the muscle cells to produce themselves the *antisense oligoribonucleotides*, AONs, which are necessary for exon skipping, so that they do not have to be applied repeatedly. This can be achieved by transporting into the muscles the genetic information for the construction of the AONs. Their idea was to use *U7-snRNAs*, small nuclear RNAs, which have a structure similar to splicing factors. These U7-snRNAs can be modified so that they are able to cause exon skipping.

To provide the *proof of principle*, the French scientists tested this approach in the mdx mouse whose dystrophin mutation, a premature stop codon, can be corrected by skipping exon 23. To achieve this, the scientists added the short DNA sequence with the information for two anti-sense sequences to the sequence of the gene for the U7-snRNA. It is important to know at this point, that the snRNAs, like all other RNAs too, are also "made" by genes. These additional DNA-sequences in the U7-snRNA gene were 24 and 20 nucleotides long and were designed in such a way that, after they are copied into RNA, can attach themselves specifically to two sequences of the mouse dystrophin pre-mRNA. One is located at the end of intron 22 and the other at the border of exon 23 with the following intron 23.

To transfer the modified U7-gene, U7 SD23/BP22, into the muscles of the mice, it was inserted together with additional control sequences into a vector, into *adeno-associated viruses*, AAV, of a type-2 genetic structure with a type-1 protein shell, parts of whose own genetic material was removed to make room for the gene sequence to be transported. Up to 20 trillion (20×10^{12}) of these modified and harmless viruses were injected into muscles of 37 mdx-mice. After six weeks, up to 80% of the fibers of the treated muscles had new shortened dystrophin which did not contain any more the 72 amino acids determined by the normal sequence of exon 23. And this new dystrophin was still present one year after this single injection of the vectors. The new dystrophin had also migrated to its normal position underneath the muscle cell membranes, and the "rescued" muscle cells looked quite normal under the microscope. The dystrophic processes in the mdx muscles, that is, their accelerated degeneration and regeneration, were completely halted. And there was also no immune reaction against the new dystrophin.

Then, five other mdx-mice were treated similarly, but the virus vectors were injected systemically into their blood circulation. After one month, more than 80% of the fibers of all investigated leg muscles had new dystrophin, and also other proteins of the dystrophin complex, which were analyzed, had reappeared. This means, that the binding sites of the new dystrophin to these proteins had the normal structure. Their muscle function was studied by measuring the spontaneous contraction of the treated muscles after they were forcefully lengthened. This normally much reduced function of the mdx-mice had returned to normal, if the muscles contained more than 70% fibers with new dystrophin. And treated mdx-mice, which were physically stressed by downhill running on a treadmill, did not develop the usual muscle damage found in non-treated mdx-mice.

This U7-gene transfer technique was then applied to treat the *dystrophic* golden retriever *GRMD-dog*. In contrast to the mdx-mouse, which is not significantly handicapped by the absence of its dystrophin, the dystrophic dog has severe clinical symptoms similar to boys with Duchenne dystrophy. Its dystrophin gene has a point mutation in the splice receptor region of exon 7 so that this exon is deleted and the reading frame after the deletion is shifted leading to a premature stop sign with the result that the dog has no dystrophin in its muscles. By skipping the two flanking exons 6 and 8 simultaneously, the reading frame can be repaired.

This is a mutation that is difficult to deal with. At first,

the required two-exon-skipping was tried with the simultaneous transfer of two different modified U7 genes against exons 6 and 8. When the results were disappointing, the researchers used in the same vector the modified gene against exon 8 and a modified gene against the two exons 6 and 7. After two months of the local intramuscular injection of 1 trillion (10^{12}) of these "tandem" vectors, a practically normal dystrophin level in the biopsy material of about 1 cm length was obtained.

This showed that there is a chance to rescue all skeletal muscles with this type of gene therapy. Therefore, as the next step, a systemic application of the vectors should have been attempted. However, the French scientists did not have enough of the viruses, loaded with the U7-RNA and the AON sequences, to treat the entire dog. Therefore, they blocked the blood circulation in one leg and injected into its veins within 15 minutes practically all of their 100 trillion (10^{14}) prepared viruses. Some oedemas developed, but that was not bad, because then the viruses migrated without problems to where they were supposed to go. Quite a lot of new and shortened dystrophin appeared, which, however was not distributed evenly in the leg muscles, but which was there in amounts similar to what Becker patients have. After six months, it was still there!

Now, a phase-I clinical trial with Duchenne boys is being prepared in which exon 51 will be skipped. Such a treatment with virus vectors will probably need immune suppression. And Luis García and his colleagues are trying also to combine this treatment with a similar gene transfer in which the gene for myostatin will be blocked. This combination was already tried in mice, although they are not the most appropriate animals, since they regenerate their muscles quite efficiently. Now, it is being tried in dogs. The results should be reported within about one year.

Clinical dystrophin transfer trial with plasmids in France: *Serge Braun*, the Director of Research and Development of the French Muscular Dystrophy Association AFM, who was not present at the London meeting., sent the following report

The AFM and the company Transgène in Strasbourg started a cooperation in 1995 to test the dystrophin gene transfer with plasmid vectors. For this technique, the combined 79 DNA exon sequences of the normal dystrophin gene, its cDNA, and its controlling structures were inserted into the genetic material of plasmids. Plasmids are small circular DNA structures without proteins inside bacteria to which they mostly confer resistance against antibiotics. As the plasmids consist only of genetic material, *naked DNA*, but do not contain any protein, no immune reaction develops against this vector and its charge.

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

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

The next step is to start a clinical trial with Duchenne patients using this new delivery system, the regional intravenous injection into single limbs under pressure. The same delivery system has been used recently and successfully in mice and dogs for exon-skipping after AAV transfer of the U7 gene in the laboratory of *Luis García*. But for the preparation of the plasmid trial, there have been some technical difficulties related to the large-scale production of the plasmid vectors with dystrophin genetic material. However, this is mainly a technical and not a conceptual problem, and that will be solved soon.

The American and French regulatory authorities have been approached in view of the new trial. Preclinical controlled safety studies are required in monkeys before the plasmid vectors can be applied to humans. Transgene and Mirus have now good records of safety in both dystrophic GRMD dogs and monkeys, so those safety studies should also not be a problem.

This plasmid full-length dystrophin approach needs to be further developed, as even in case exon skipping becomes successful in humans, patients who are not eligible for exon skipping need alternatives.

Myogenic cell transfer, clinical trials in Canada:

Jacques Tremblay of Laval University in Québec City, who was not present at the London meeting, sent publications about new experiments with the myoblast transfer technique, which now should be called *transplantation of myogenic cells*.

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

At the moment, a second clinical trial is underway with injections throughout a muscle in which one would be able to demonstrate easily a functional improvement – lifting the hand using the wrist only – . This cell transplantation would have some advantages, among them, (1) the new dystrophin would have the normal length and be under the control of its normal control sequences; (2) the positive effect would be long-term; (3) the technique could be combined with a myostatin inhibition; and (4), most importantly, it could also help older Duchenne patients. However, the human ethic committee of his hospital has limited the participation of the patients only to those that are more than 18 years old. This is a major restriction making difficult the recruitment of patients that can still do this motion on both sides. Indeed one side is injected with myogenic cells while the other side is injected with saline. The patient does not know which side has been injected with cells so that the improvement can be evaluated without bias.

Several parents have concerns about the large numbers of injections required to deliver the myoblasts throughout a muscle – 100 injections per square centimeter –. This type

of transplantation has so far been done in a large number of monkeys without problems and more recently in two DMD patients under only local anesthesia which was enough to reduce the local pain felt after the end of the local anesthesia and both patients said that they would receive additional injections in other muscles without hesitation. In collaboration with AFM, Dr. Tremblay has organized a small meeting in Evry for the beginning of February 2007 to understand the mechanisms involved in myoblast migration in a muscle so that the number of injection could be eventually reduced.

The group of Dr. Tremblay is also pursuing research work on blocking myostatin in combination with the transplantation of myogenic cells. Recent results in dystrophic mice clearly demonstrate that the muscle fibers expressing dystrophin are more than twice bigger. The presence of dystrophin should make the muscle fibers more resistant during muscle contraction while their increased size should increase the strength of the muscle.

Another research topic is the development of immunological tolerance. This is a treatment which aims at avoiding the requirement of a sustained use of immunosuppressive drugs following myoblast transplantation. The results obtained in mice are excellent. Dystrophin positive fibers were obtained in the dystrophin muscle without a sustained immune suppression and it was even possible to do a second transplantation of myogenic cells without repeating the tolerigenic, immune suppression, treatment. Similar experiments are ongoing in monkeys and dogs to obtain results that may permit one day to use such tolerigenic treatments in patients. There are still, however, several years of research before this goal is met. This tolerigenic treatment would eventually be useful for the transplantation of stem cells obtained from a donor, such as the mesoangioblasts currently investigated by *Giulio Cossu*.

Gene transfer with mesoangioblasts, adult muscle stem cells: *Giulio Cossu* of the Stem Cell Institute at the Hospital San Raffaele in Milan, who was not in London either, updated and amended my earlier summary on his work:

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 exclusively to muscle cells and to nothing else, especially not to tumors. And it should be possible to apply these cells systemically by injecting them into the blood vessel system which could distribute them around the body. Then they have to cross the vessel wall, and stay inside muscle 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 the muscle tissue.

Giulio Cossu and his colleagues performed basic experiments whose results will not only become important for a stem cell treatment of Duchenne dystrophy but also for many other muscular dystrophies.

The Italian researchers did not use the mdx mouse as an animal model but a mouse whose gene for alpha-sarcoglycan, one of the proteins of the dystrophin associated complex, was made inactive. These mice have a type of limb girdle muscular dystrophy, LGMD, which is clinically similar to Duchenne dystrophy. The researchers isolated mesoangioblasts from normal mice, which contained the intact alpha-sarcoglycan gene, treated them with several

different growth factors and then injected them into the blood circulation of the LGMD mice. These "healthy" stem cells were able to migrate into all skeletal muscles of the living mice and caused the re-appearance of more than 80% of the normal amount of alpha-sarcoglycan. Similar results were obtained with mesoangioblasts isolated from dystrophic muscle and transduced with a viral vector expressing the sarcoglycan gene.

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

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' hindleg. Before infusion, the gene, the cDNA, of a micro-dystrophin was transferred with a lentiviral viral vector into the dystrophic mesoangioblasts.

Up to five monthly injections of either the normal or corrected dystrophic mesoangioblasts led to new dystrophin protein in many muscles in the dystrophic dogs. In one animal, the cells were released from a catheter into the aorta. This allowed more widespread dissemination of the mesoangioblasts. 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. In general, dogs receiving donor cells from normal animals improved more than those receiving the corrected mesoangioblasts from the same dogs. Some muscles in the injected dogs had nearly normal levels of dystrophin, and even the muscles with only moderate levels of dystrophin showed significantly improved structure and function.

With further experiments, the researchers were able to increase the therapeutic efficacy of the mesoangioblast by treating them in cell culture either with DETA-NO or

IMN, two well-known compounds which release the gas NO, nitric oxide, into the culture medium. Nitric oxide acts like a hormone, its main effect is a dilation of blood vessels. After the systemic injection of the NO-treated mesoangioblasts into the arteries of the LGMD mice without alpha-sarcoglycan, these stem cells had a significantly enhanced ability to migrate to dystrophic muscles, to resist their cell-destroying environment and to fuse with regenerating muscle fibers. This resulted in a significant recovery of the production of alpha-sarcoglycan. This treatment thus was able to limit muscle damage of the dystrophic mice.

In view of the ultimate aim to develop a Duchenne therapy, the scientists had the idea to look for a drug which not only could generate NO but also had anti-inflammatory properties similar to prednisolone. As the drug of choice, HCT 1026 was selected, a derivative of flurbiprofen, INN, one of the most potent nonsteroidal anti-inflammatory drugs. HCT 1026 releases NO and does not show the often severe side effects of corticosteroids. It is a known and safe drug, already approved for human use, and effective on oral administration. Thus it is suitable for long-term treatment. The anti-inflammatory action of HCT 1026 does not correct the genetic defect of Duchenne dystrophy, but its ability to generate NO, which had been shown to stimulate the effect of mesoangioblasts, suggested that a combination of these two approaches could be especially promising.

Therefore, *Giulio Cossu* and his colleague *Emilio Clementi* and their teams tested the effect of HCT 1026 alone and together with mesoangioblasts on the two animal models of muscular dystrophy, the alpha-sarcoglycan-negative and the mdx mouse in a long-term trial lasting one year. HCT 1026 alone dramatically slowed the progress of the disease and maintained muscle integrity and function. It was significantly more potent than prednisolone and did not induce detectable side effects. Moreover, the combined drug and stem cell treatments increased up to 4-fold the number of dystrophin-positive fibers in mdx mice and thus improved significantly the therapeutic potential of mesoangioblasts by increasing their migration to and fusing with dystrophic muscle fibers. These results open a window to an effective therapy of Duchenne muscular dystrophy.

Pharmacological approaches.

Utrophin to replace dystrophin: *Kay Davies* of Oxford University is one of the "giants" who in the 1980s worked like *Louis Kunkel* on finding the gene which causes Duchenne muscular dystrophy when inactivated by a mutation. They were then and still are in a friendly competition to find a treatment, they exchanged students and, as is usual among scientists with the same goal, they meet often, discuss their work and their ideas and plans and thus stimulate each other's work. The research field of muscular dystrophy has produced remarkable results in the last years so there will be several effective treatments for this terrible disease.

There are four great challenges that have to be met by anybody trying to find a therapy for Duchenne dystrophy: (1) Dystrophin is a huge protein whose function has to be restored; (2) to make a difference, at least 20% of the nor-

mal 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 Dr. Davies and her colleagues tried to find the Duchenne gene, they found instead this other protein, *utrophin*, and its gene, and they started to use simple compounds as drugs to upregulate the low amount of this protein in muscle tissue. Such drugs may have side effects but because they are small molecules and not proteins, they do not produce immune problems, and will be easy to deliver into the blood circulation to reach all muscles.

Utrophin is a protein with a structure and function very similar to dystrophin. In humans, its gene is located on chromosome 6, it has 75 exons, and is about one million

base pairs long. The utrophin protein is about 7% shorter than dystrophin. 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 muscle, but there it is concentrated in regions where the motor nerves contact the muscle membranes, the *neuromuscular junctions*.

If utrophin and dystrophin are so similar, why does nature not use utrophin when dystrophin is missing in Duchenne patients? There are indications, that nature really tries this. 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 is an indication that upregulation of utrophin would make Duchenne dystrophy more benign. During development at 12 weeks, the muscles have both, utrophin and dystrophin, then utrophin disappears from the cell membranes, remains only at the neuromuscular junctions, and at birth dystrophin alone remains on the membranes. Thus utrophin is a fetal form of dystrophin. This means, that if one could reactivate the developmental program for utrophin, one would get a treatment for Duchenne dystrophy.

Mdx mice whose utrophin gene was *knocked out* experimentally, which thus 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 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 could be 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 dystrophic symptoms could be prevented and this led to a complete functional recovery.

Thus, for a possible Duchenne therapy, one should try to increase the low amount of utrophin by *upregulation* of the activity of its gene. The gene is now well known, but 20 years ago, it took two years to find and characterize it, today that could have been done in two weeks. The gene has two different start sites, promoter sequences, to which signaling compounds bind to initiate the synthesis of the utrophin protein. One promoter induces the production of one form of utrophin, the A-form, in the regions of the neuromuscular junctions. The other promoter starts to make the other form, the B-utrophin, in the blood capillaries. One part of the promoter sequence, the N-box, contains a stretch of six nucleotides, TTCCGG, that seem to be responsible for confining the A-utrophin to the muscle-nerve junctions. The researchers are now trying 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.

Even before all these molecular details of the utrophin biology was elucidated, work started to find a substance that could upregulate utrophin and properly direct it to the membranes. That could not be done in a university laboratory. So *Kay Davies* looked for a pharmaceutical company that would be interested in this difficult and expensive task, and as she could not find one, she herself founded a new one: VASTox plc. in Abingdon near Oxford.

Jon Tinsley of this company then reported in a separate presentation how much has been done to find a potential utrophin drug: Until now, 13,000 chemical compounds have been screened for their ability to upregulate the activity of the utrophin gene in mdx mice. The light-producing enzyme luciferase from fireflies was used in a reagent system to test for the presence of utrophin. More than 100 promising substances were found which could increase the low utrophin concentration three- to fourfold. They are now being optimized and tested on muscle cell cultures and 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.

One of the most active compounds, VOX185, has already been tested systemically in mice by injection into the abdomen. It reacts only with the promoter of the A-form of utrophin, that is present in muscle. The A-utrophin in all of the skeletal muscles of the mice tested could be upregulated two to threefold, but it is not known yet, whether the utrophin in cardiac muscles is upregulated also. After 12 weeks of weekly systemic injections, the animals showed a significant recovery of their muscular function.

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

Steroid therapies, international clinical trial: In her second presentation, *Kate Bushby* discussed the use of steroids in Duchenne dystrophy and the planned international clinical trial with prednisolone.

For about the last 20 years, the corticosteroids *prednisone*, the very similar *prednisolone* and the newer form *deflazacort* have shown positive effects in Duchenne boys: The daily use of these drugs preserve the ambulation of the boys over several years up to about their mid-teenage years; they increase their energy, the function and the power of the patients markedly, so that they can better take part in social activities; they reduce the need for a surgical correction of scoliosis, the curvature of the spine; they preserve significantly the vital capacity, the lung function; they seem to be cardioprotective, meaning that they have also a positive effect on the heart; and the most positive results have been shown in the younger boys. The usual doses are 0.75 mg/kg/day for prednisone and prednisolone, and 0.9 mg/kg/day for deflazacort. However, the drugs may also have side effects: weight gain so that the drugs can make the boys hungry and lead to Cushingoid features, a round face; osteoporosis, weakened bones with an increased risk of fractures; cataracts, a slight opacity of the eye lenses, especially with deflazacort; and reduction of height by slowing their growth.

Although the main medical effects of corticosteroids in other diseases are a reduction of inflammation and an effect on the immune system, this does not seem to be an explanation why prednisone, prednisolone, and deflazacort have positive effects in improving strength in most boys with Duchenne muscular dystrophy. And because the side effects discourage many families to use these drugs, at least 15 different application methods, which are supposed to minimize the side effects, are in use in the treatment centers worldwide. Dr. Bushby now works in this field for the last 17 years, she realizes that the situation is still "chaotic" and that a large, if possibly international, clinical

trial is needed to determine once and for all the details of an optimized treatment regime.

Therefore, in the UK, 19 centers have signed up to take part in the *North-Star Project* which is working to a consensus on the use of these steroid drugs taking into account all the knowledge accumulated over the years about the long-term positive and negative effects of prednisone and its other forms for maintaining the muscles of Duchenne boys. The results of this cooperation will provide the basic data for the planned large international clinical trial which already has a name: FOR-DMD.

More than 50 centers in 12 countries will perform this trial over the next years with at least 300 Duchenne boys under the supervision of the *FOR-DMD Trial Managing Group* in which many internationally known scientists and clinicians will participate. The most important aims of the trial will be: (1) Testing daily prednisone/prednisolone versus daily deflazacort versus intermittent prednisone/prednisolone regimes on the hypothesis that the daily application will be more efficacious on the long-term maintenance of respiration, the time to rise from the floor, and patient satisfaction; (2) determining the tolerability of the different regimes and their side effects; and (3) initiating additional studies on protein and polymorphism profiling to find out why steroids work. The National Institutes of Health, NIH, in the United States will probably fund this international study. The Managing Group will file the full grant submission in 2007 after a planned meeting in the Netherlands.

Despite their side effects, there is still a major role for steroids in Duchenne dystrophy. Although the approaching genetic therapies will be available in the not too distant future, an optimized steroid treatment will be the gold standard against which other treatments can be judged. And because the genetic drugs will be expensive and not easily affordable in developing countries, the much cheaper steroid drugs will have to be used for many more years there.

Kate Bushby finished her presentation by saying that the majority of the children treated with prednisone show an overall benefit from using the drugs and that the natural history of the condition has changed since the use of corticosteroids has become widespread. Together with the orthopedic, cardiac, and respiratory care already available, the use of steroids in an optimized regime with careful attention to possible side effects will continue to make a huge and positive difference in the life of Duchenne boys.

Reading through premature stop codons with PTC124.

Richard Finkel of the Children's Hospital of Philadelphia, a Principal Investigator on the PTC124 study, presented preliminary data from the Phase-IIa studies. In his presentation, Dr. Finkel updated the information given three months earlier by Dr. *Langdon Miller*, Chief Medical Officer of PTC Therapeutics, South Plainfield, NJ. at the Parent-Project meeting in Cincinnati. Therefore, some of the following summary uses parts of the text given in the report on the Cincinnati meeting.

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

fully assembled. These mutations are called nonsense mutations because they do not lead to the production of an amino acid chain of the protein dystrophin. These mutations are also known as stop mutations or premature termination codons.

PTC124 is an oral drug designed to overcome these nonsense mutations. It has been developed in a drug screening program conducted by PTC Therapeutics. It is a novel, first-in-class drug, which is not like any other drug. It helps the cellular machinery to overcome one of the genetic causes of Duchenne dystrophy but it is not gene therapy or an exon skipping. Although the antibiotic gentamicin was shown to act in a similar way, PTC124 is not related to gentamicin and is not an antibiotic. PTC124 is not yet available commercially, it is still under development in clinical trials. It is a white crystalline powder that is mixed with water, milk or juice.

Because PTC124 targets the nonsense mutation in the mRNA, not the gene, it has the potential to treat other hereditary diseases, among them cystic fibrosis. PTC Therapeutics is conducting Phase-II studies in cystic fibrosis as well. PTC124's action is not disease-specific but mutation-specific, it is not a potential drug for Duchenne dystrophy alone, but a potential drug for all genetic disorders due to a nonsense mutation.

Only those 15% of Duchenne boys who have the disorder due a nonsense mutation can potentially benefit from PTC124 treatment. Therefore, it is necessary to know the exact mutation of a Duchenne boy in order to determine whether it is a nonsense mutation. Only when that is true, PTC124 could be a drug for him.

To determine whether PTC124 could be a Duchenne drug, preclinical experiments were done on cell cultures and mdx mice. Full-length dystrophin appeared in living animals after oral dosing with a positive effect in muscle fibre resistance to stretch induced injury. In experiments done in rats and dogs, no read-through of normal stop codons was detected. Toxicity studies in rats and dogs given high doses of the drug have generally not shown serious, acute side effects. These data showed that clinical tests on Duchenne patients were justified.

In the first clinical testing of PTC124, two phase-I trials were performed in 61 healthy 18-to-30-year old adult volunteers. In these studies, the drug appeared safe and demonstrated few side effects. Doses of up to 100 mg/kg/day were well tolerated by these healthy adults, a dose which is larger than planned to be given to Duchenne boys. These results supported the initiation of a Phase 2a clinical trial.

The initial part of this phase-IIa clinical trial on 26 Duchenne boys, 5-13 years old, has now been completed. Six boys received a dose of 16 mg/kg/day and 20 boys 40 mg/kg/day of the drug in three portions per day. They had different stop mutations: 19 had stop codon UGA, 5 UAG, and 2 UAA, distributed from 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 after an additional 28 days. Muscle biopsies were performed before and after the treatment on the *extensor digitorum brevis*, EDB, foot muscles to check for the partial restoration of full-length dystrophin production. Other chemical and functional tests were also done to measure the therapeutic effect and monitor for safety.

Very preliminary data showed that in cultured muscle

cells, obtained from the initial biopsy material then exposed directly to PTC124, the expected full-length dystrophin was detected. The muscle biopsies performed after taking the drug for 28 days demonstrated slight visible increase in dystrophin in some of the boys. In this study, as before in the phase-I trial, no serious side effects were observed. But the level of the new dystrophin was still too low to produce a sufficient and reliable therapeutic effect. However, several parents and teachers observed that, after the treatment, the boys showed greater activities, increased endurance, and less fatigue than before the treatment. These are not really scientific results, they have to be evaluated in more detail.

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 microgram/ml of plasma, due to the possible faster degradation of the drug in children when compared to adults. Longer exposure to the drug may also be necessary to achieve better dystrophin expression. Therefore, the Phase 2a trial has been modified to enroll an additional 12 Duchenne boys, who will receive 80 mg/kg/day PTC124 for 28 days, aiming for a blood concentration of at least 2 to 10 microgram/ml in plasma. The final data will be reported in 2007. PTC Therapeutics plans to initiate longer-term studies lasting three to six months in late 2007.

Upregulation of IGF-1: *Nadia Rosenthal* of the European Molecular Biology Laboratory in Monterotondo near Rome described the influence of the natural growth factor IGF-1 on muscles and the role it could play as a therapeutic agent for children with Duchenne muscular dystrophy.

IGF-1, insulin-like growth factor, is a protein of about 70 amino acids in one chain with three stabilizing bridges, thus with a similar shape as insulin. It exists in multiple forms with slightly different structures. The effects of some of these different forms depend largely on where they are produced in the body. One of these so-called isoforms, mIGF-1, which promotes cell growth in muscle tissue, is of interest for a possible therapeutic use in Duchenne children.

mIGF-1 promotes the regeneration of muscles very well and helps to form functional muscle because it activates the synthesis of new protein. Local injury of muscle results in a burst of mIGF-1 production, therefore, it is a good candidate for a combination with other possible Duchenne therapies. It could keep the muscles in good shape while the genetic damage is being fixed by genetic techniques.

In order to answer the question of what will happen when the amount of mIGF-1 in the muscles is increased above the normal level, transgenic "normal" mice were created which had no dystrophy but which had extra IGF-1 genes in the nuclei of their muscle cells. These *non-dystrophic* mice with high concentrations of mIGF-1 showed a strong muscle hypertrophy, enlarged fibers, after 14 months. Some of the other effects were a decrease of fat, the extended maintenance of muscle mass and strength in aging animals, and an accelerated healing of muscle injury. The additional mIGF-1 did not induce cardiac problems, did not promote cancer, and had no pathological side effects. *Dystrophic* mdx mice, in which also transgenic additional IGF-1 genes were produced, showed decreased inflammation and fibrosis, and much reduced muscle degeneration. Thus mIGF-1 improves the local environment for

efficient muscle regeneration.

The question was now, whether one of the other isoforms of IGF1 works better than mIGF-1. The coding sequence of IGF-1 is relatively small, its few exons rearrange on the way to its mRNA by alternative splicing, producing different extension (E) peptides. The mIGF-1 isoform contains an Ea variant, which is mainly active in skeletal and cardiac muscle. Ea means that this form has an extension signal peptide of about additional 35 amino acids. A student in Dr. Rosenthal's team worked three years to make transgenic animals with each of the four more important isoforms. Only the forms with the additional Ea peptide increased muscle mass and strength promoted regeneration. Tests with isoforms containing other E peptides showed that they only improved regeneration. That means, one does not have to induce a muscle mass increase to be able to improve and to maintain muscle regeneration. E peptides are very important for IGF-1-mediated regeneration – an artificial form lacking an E peptide did not have any beneficial effect on muscle.

So the muscle regulating system, in which IGF-1 participates, is much more complicated than was originally thought. Possibly there are so-called binding proteins on the outside of the muscle cell which stabilize the mIGF-1. If the EA peptide is present, the mIGF-1 promotes muscle mass growth and regeneration. If the EA peptide is absent, then there is still regeneration but no increase of muscle mass. However, the details of this process are still not completely understood. However, the full understanding of the action mechanism will be extremely important before IGF-1 can become a therapeutic drug for children with Duchenne dystrophy.

But it will always be an adjunct therapy, because it is not acting on the genetic cause of the disease. One will probably have to combine growth factor therapy with stem cells, AONs, myostatin inhibitors, or other small molecules. If one could synthesize in the laboratory mIGF-1 with a stable EA peptide, that may lead to the appropriate therapeutic approach. Ideally, delivery of this most effective form into the muscles could be achieved without having to transfer its gene, that is, without genetic procedures. Just injecting the purified compound into the blood stream or directly into the muscles, or eating the undefined mixture of the different forms as offered on the internet, will be ineffective or even dangerous.

An interview with *Nadia Rosenthal* on the hope the parents should not lose while waiting for a genetic therapy can be seen in my report on the meeting in Monaco in January 2005 at my internet pages www.duchenne-research.com.

Inhibition of myostatin: This summary starts with an introduction to the substantial previous knowledge about the nature and action of myostatin – as described in my earlier reports – and then summarizes the new work done by *Ian Graham* in *George Dickson's* laboratory at the Center for Biomedical Sciences, Royal Holloway, of the University of London.

Myostatin is produced in muscle and other organs and circulates in the blood as an inactive protein consisting of 375 amino acids. To become biologically active, the first 266 amino acids, called the *propeptide*, are split off, and two chains of the remaining portion with 109 amino acids each combine to form a double ring. The separate propep-

tide again attaches loosely to the double ring, still inactivating it. The propeptide is removed and quickly degraded when the complex binds to its receptor *activin* on the outside of the muscle cell membrane. This *myostatin-activin* complex then initiates on the other side of the membrane, inside the cell, a series of chemical reactions, a signaling cascade, that finally interrupts the genetic regulation that would otherwise lead to the biosynthesis of new muscle proteins. This process thus inhibits muscle growth. Therefore by inactivating myostatin, the regeneration of the muscle fibers of Duchenne boys could possibly be stimulated so that they would not be destroyed as fast or might even increase in size.

Non-dystrophic mice whose gene for myostatin had been knocked out by genetic methods, have up to three times larger skeletal muscles with significantly more fibers of larger than normal diameter, they have undergone *hypertrophy*. There are cattle, the *Belgian Blue Breed*, which are very muscular because their myostatin gene was inactivated by a mutation centuries ago. And in Berlin, a now 8-year old boy was identified whose skeletal muscles are about twice as large as in a normal child. Because a mutation in this family had changed the normal splicing of the three myostatin exons, the boy has a very low level of myostatin in his muscles. This is a strong indication that the inhibition of myostatin would lead to an increase of muscle growth in Duchenne boys, too.

The *Wyeth Research Company* in Collegeville near Philadelphia developed a specific antibody, MYO-029, against myostatin which has been shown in animal studies to increase muscle mass significantly. In humans, it does not cause immune rejection because the protein structure of the antibody is the human one, it is "humanized". It can be injected into the circulation or under the skin. In cooperation with *Kathryn Wagner* in Baltimore and *Lee Sweeney* in Philadelphia, Wyeth has now tested in a phase-I/II clinical trial this potential antibody drug in 108 adult muscle disease patients, among them some Becker patients. The trial is now complete, the results, which seem to be positive, will be published in the spring of 2007.

George Dickson and his colleagues, in collaboration with *Wyeth* have started another approach to inhibit myostatin. After the propeptide is released when the active part of the myostatin binds to the receptor, this isolated chain of 266 amino acids is unstable, it is degraded in the bloodstream with a half life of only two hours. The scientists then had the idea to transfer the gene for the propeptide into muscles with the aim to maintain this natural inhibitor of the action of myostatin permanently at a high level. They tried this approach with normal mice and found that the best vector for this gene transfer was the type-8 adeno-associated virus, AAV8, which is able to infect non-dividing muscle cells. After the local injection of the vector construct into the tibialis anterior muscle of these non-dystrophic mice, they obtained a muscle mass increase of 30% at 4 weeks which rose to 40% at 10 weeks. The systemic injection into the blood circulation increased the mass of the same normal mice by 25%, and this increase was maintained for 10 weeks. The muscle force was also improved, even in the slow muscle fibers. If these results could be reproduced in Duchenne boys, this method would keep them out of the wheel chair longer.

All these results were obtained in preliminary experiments with normal mice. They have to be repeated now

with mdx mice. Clinical trials with patients could follow if the results with mice and other animals continue to be positive. 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.

SNT-MC17/idebenone in clinical development: *Thomas Meier*, Chief Scientific Officer at Santhera Pharmaceuticals in Liestal near Basel could not come to the meeting in London. He sent the following summary:

SNT-MC17/idebenone is a molecule that protects mitochondria, the power stations in the cells where the universal energy carrier, adenosine triphosphate, ATP, is made by oxidative phosphorylation. This compound SNT-MC17, or *idebenone*, has recently successfully completed a clinical trial in the US in collaboration with the NIH demonstrating efficacy on neurological aspects of Friedreich's Ataxia, another devastating neuromuscular disease. A phase-III clinical trial for Friedreich's Ataxia is already ongoing in Europe. Friedreich's Ataxia is a rare neuromuscular disease which besides neurological symptoms is frequently associated with cardiomyopathy, a severe disease of the heart muscle.

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

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

This trial is called Duchenne Efficacy Study in Long-term Protocol of High dose Idebenone, DELPHI. Its results will be available in the second half of 2007.

Clinical trial with prednisone and cyclosporin: Under the direction of *Rudolf Korinthenberg* at the Children's Hospital of the University of Freiburg, who was not in London, a clinical trial with prednisone and cyclosporin is being performed in Germany.

At present, the two cortisone preparations prednisone and deflazacort are the only drugs known which bring a noticeable improvement for boys with Duchenne dystrophy. But the prednisone treatment has side effects. Therefore, some clinicians in a number of German muscle centers have decided to reduce the total prednisone dosage by

giving the normal dosage not continuously but only 10 days in a row and then interrupting the treatment for 10 days so that one still gets the protective effect but avoids at least some of the side effects. However, it turned out that the therapeutic effect is somewhat smaller than when prednisone is given continuously daily. In order to improve this situation, it was suggested to combine the prednisone treatment with cyclosporin. Cyclosporin is a drug which reduces the immune reactions. It has a different mechanism of action from cortisone. It also has side effects, but during a long-term treatment, they are somewhat more favorable than those of prednisone.

The clinical trial with the two drugs started in Germany at the beginning of 2004 in which one half of the patients receives 3.5 – 4 mg/kg/day cyclosporin combined with 0.75 mg/kg/day prednisone and the other half the same prednisone dose alone. Each patient participates in the study for 15 months. At the moment, in January 2007, 150 patients are being treated or have already completed their treatment. A few more will be added during February. A minimum of 150 patients was needed to evaluate the study correctly. The study will continue through 2007 and part of 2008, so that the results can be analyzed next year.

No results about the combined effects of cyclosporin and prednisone are yet available, because the trial is a double-blind study, i.e., neither the patients nor the scientists know whether a particular child has received cyclosporin or a placebo, a neutral substance, in addition to prednisone. But we can say that the study is functioning very well and that no severe side effects have appeared. Of the 150 patients, only two had to leave the study, one, because he lost his ambulation relatively soon, and the other, because a diabetes manifested itself which we had not discovered before his enrollment.

Oxidative stress and Duchenne dystrophy: Joe McCord of the University of Colorado and the company LifeVantage Corp. in Denver discussed a new way of therapeutic control of oxidative stress. Oxidative stress is an important component of more than 100 diseases, among them Duchenne dystrophy.

Every cell, also each muscle cell, contains many mitochondria. They are oval-shaped organelles typically about 0.002 mm in length and 0.0005 mm in diameter, about the size of a bacterium. They are the power stations of the cell because they synthesize the energy-rich compound adenosine triphosphate, ATP, by the process of oxidative phosphorylation. The end products of this energy production are carbon dioxide and water. But about 1-2% of the oxygen consumed is not converted to water, but rather to the *superoxide free radical*. It has an odd number of electrons which makes it especially reactive. The cell defends itself against this toxic product with two very efficient enzymes. One is *superoxide dismutase*, SOD, which converts the radical to hydrogen peroxide, that is less reactive but still an oxidant. The other enzyme is *catalase*, which converts the hydrogen peroxide into water and oxygen, which are completely non-toxic.

When the production of the superoxide radicals exceeds the normal limit of 2%, the cells experience *oxidative stress*. This happens any time a cell is injured. Muscle cells become dystrophic when large amounts of calcium ions enter through the cell membrane into the interior where they cause the mitochondria to swell and to disrupt with

the consequence that more than normal superoxide radicals are made, more than the cell can destroy. The excess radicals can oxidize lipids, oxidize proteins, modify enzymes, damage DNA and cause even cancer. They are also contributing to the aging process.

Another source of oxidative stress is inflammation. White blood cells are the only cells that make free radicals intentionally in order to kill and digest invading microbes and viruses. But this process also damages the host cells, and cause muscle cells to release proteins into the blood stream like, for instance, creatine kinase. Inflammation also changes the signaling processes and may cause apoptosis, the self destruction of cells. Also fibrosis is triggered by oxidative stress, the forming of scar tissue in diseases with chronic inflammation, among them Duchenne dystrophy where elastic muscle tissue is replaced by inflexible connective tissue.

Twenty years ago, it was found that the most prevalent products of oxidative stress, the peroxidized lipids, were increased by an average of 35% in Duchenne boys. But until very recently, it was impossible to restore the oxidative balance when, in 2006, *Werner Boecker* and his colleagues in Germany showed that in Duchenne and Becker patients, the muscle fibers and the vasculature, the blood vessels, undergo massive oxidative stress resulting in a reduction of bioactive NO, nitric oxide, a “good” free radical. This gaseous hormone is produced by the enzyme NO synthase, NOS, and regulates, among other processes, the elasticity of blood vessels, the vascular tone. The enzyme NOS is upregulated in muscle tissue which tries to normalize the amount of NO, but this process is insufficient because of the muscle degeneration and also because NO reacts with the superoxide radicals. The product of this reaction is peroxynitrite, OONO⁻, another reactive oxidant which intensifies the oxidative stress.

In earlier laboratory experiments, it was shown that the addition of the enzymes superoxide dismutase and catalase to isolated perfused hearts could block the oxidative stress by destroying the excess of the free radicals and prevent the release of the enzyme creatine kinase. In other studies, superoxide dismutase was shown to reduce the elevated levels of the protein factor TGF-beta which is a signal for fibroblasts to produce fibrosis.

The muscles of Duchenne boys function relatively well until about three years of age. After this time, the consequences of oxidative stress overcome more and more the normal muscle regeneration. These consequences are chronic inflammation, fibrosis and also cognitive impairment. For a therapy of Duchenne dystrophy, it would be important to interrupt these processes at this age or earlier.

It was believed that one rather easy way to do this would be the ingestion of the known antioxidants vitamin E and vitamin C. But it had been shown in many studies, that large quantities of these vitamins do not have an effect on oxidative stress. Also eating 600 grams of fruits and vegetables per day had no effect on oxidative damage to DNA. The “antioxidant” vitamins are important for other reasons, but they do not reduce oxidative stress. The other possibility is to increase the level of the two enzymes superoxide dismutase and catalase which, as mentioned, can destroy the excessive amount of free radicals. After all, one molecule of vitamin C can eliminate two molecules of free radicals, but not more, whereas one molecule of superoxide dismutase can eliminate one million free radicals

every second and maintain this activity for a long time.

Thus Dr. McCord with the company LifeVantage developed a dietary supplement containing “adaptogenic” compounds from five plant species for effectively inducing the two antioxidant enzymes to reduce the oxidative stress, mainly the peroxidation of lipids. This supplement, trade-named *Protandim*®, contains extracts of the plants *Bacopa monnieri*, *Silibum marianum* or milk thistle, *Withania somnifera* also known as ashwagandha, *Curcuma longa* from which the spice turmeric is derived, and *Camellia sinensis* or green tea. The last one provides one of the active ingredients of green tea, (-)-epigallocatechin gallate or EGCG, which had been shown before by *Urs Rüegg* in Geneva to have a beneficial effect on Duchenne dystrophy.

In 2006, Protandim has been clinically tested in 29 healthy persons in age from 20 to 78 years. Several parameters were measured at the beginning of the study and

after 30 and 120 days of daily supplementation with Protandim, among them superoxide dismutase and catalase. The average increase after 120 days were 30% for superoxide dismutase and 54% for catalase and the peroxidation of lipids was significantly inhibited. Important for the age-related increase of oxidative stress was the fact that after 30 days Protandim, the age-related increase of lipid peroxidation virtually disappeared, and its average level dropped by 40%.

In conclusion: While Duchenne muscular dystrophy is caused by a specific genetic defect, there is abundant evidence that oxidative stress becomes an increasingly important factor in the progression of the disease. The new developments in the effective management of oxidative stress justify immediate consideration of clinical trials with Duchenne patients.

Diagnosics and registration.

Why should one test for variations, the mutations, in the dystrophin gene? *Stephen Abbs* is the Director of the DNA Laboratory of Guy’s Hospital in London. The question in the title of his presentation is practically the same as the one used by *Kevin Flanigan* at the PPMD meeting in Cincinnati in July 2006. Therefore, the first paragraph and the description of the MLPA method is almost the same as in the summary of Kevin Flanigan’s presentation in the report on the Cincinnati meeting:

The exact mutation of a boy who seems to have Duchenne dystrophy should be known to confirm that he really has Duchenne dystrophy, and not some other muscle disease, e.g. one of the many limb-girdle dystrophies which might show symptoms similar to Duchenne. This principle is the same as that used 20 years ago, when chromosome breaks and deletions on a particular region on the short arm of the X chromosome were found in patients with Duchenne muscular dystrophy and not in unaffected individuals. This was evidence that these variants caused muscular dystrophy and enabled *Louis Kunkel* and his colleagues to identify and characterize the dystrophin gene.

This same principle is now applied to individual patients, since if one can identify a pathogenic variant in the dystrophin gene it now confirms the clinical diagnosis of Duchenne or Becker muscular dystrophy. The type of the mutation does not always distinguish correctly Duchenne from Becker muscular dystrophy. But if the mutation shows that the dystrophin reading frame is shifted, Duchenne muscular dystrophy is much more likely. Then, in most cases, a muscle biopsy can be avoided. Equally important, knowing the exact mutation allows reliable genetic counseling of the boy’s family and his maternal relatives, among whom Duchenne genetic carriers can be detected. In addition, prenatal and preimplantation diagnoses are only possible when we know which gene is responsible for the disorder. The only way to confirm this is by identifying the exact mutation in the family. Finally, new therapies, such as exon-skipping methods, and stop-codon read-through drugs require knowledge of the specific mutation within the patient’s dystrophin gene.

About 60% of the dystrophin mutations are deletions of whole exons, about 10% are duplications of whole exons, about 30% are point mutations that involve changes of

only 1 to 4 nucleotides, and about 1% are inversions, complex rearrangements, or mutations deep in the introns.

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

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

If no mutation can be found with the MLPA test, the patient probably has a point mutation, which involves just one or a few of the two and a half million nucleotides in the gene. There are two general approaches to identifying these mutations. One is to perform a preliminary scan on all 79 exons to look for any exons that show different results compared with normal results, and then just the exons showing any differences can be sequenced to find whether the cause of the difference is a normal variation within the gene, a polymorphism that does not cause a disease, or whether it is the mutation causing the muscular dystrophy. The alternative method is to sequence all 79 exons straight away, which is a more costly approach.

Because the gene is so huge, many methods for scan-

ning the exons have been developed. Dr. Abbs mentioned 9 different ones. The one which has been used most in his laboratory was developed by his colleague *Emma Ashton* and is called *fluorescent multiplex conformation sensitive capillary electrophoresis*, FM-CSCE. A similar process is now being developed at Guy's Hospital by *Annabel Whibley* and is called *temperature gradient capillary electrophoresis*, TGCE. Instruments for the development of the TGCE method were funded by the UK Department of Health for speeding up genetic testing in the UK.

The FM-CSCE method uses 12 fluorescent multiplex PCR assays which amplify 84 DNA fragments representing all 79 exons. Then the PCR products from the patient's DNA are mixed with the corresponding PCR products from an unaffected individual's DNA to generate duplexes. In most cases, the patient's DNA will be the same as the normal DNA, so both partners of such a duplex have the same structure; they are *homoduplexes*. If the partner that comes from the patient has a point mutation, the nucleotide sequences in this duplex will not match completely and the DNA duplex has a bulge at this site; it is a *heteroduplex* and migrates at a different speed in an electrophoresis compared with a *homoduplex*. This different migration enables *heteroduplexes* to be identified, and any exon from a patient's DNA that results in such a heteroduplex can then be sequenced to determine the change that is causing the heteroduplex. The change in the sequence then has to be interpreted to decide whether it is the mutation causing the muscular dystrophy, or whether it is a normal variation, a polymorphism.

If neither deletions nor duplications nor point mutations are found in a boy with typical Duchenne symptoms, then he may have a mutation that is difficult to identify and may involve a complex rearrangement, an inversion, or maybe a mutation in one of the introns of the gene. These types of mutation, which are thought to be present in about 1% of DMD patients, generally will not be detected in DNA, and RNA must be used to detect them. To find these rarer mutations in the dystrophin gene, its mRNA has to be isolated from biopsy material and investigated. *Steve Abbs* mentioned one example where a new exon with 71 nucleotides was inserted between the normal exons 44 and 45 because the gene had a point mutation far inside the intron 44 which, with 250,000 nucleotides, is one of the largest introns in the dystrophin gene. This mutation was located about 9,000 nucleotides away from exon 45, and because standard mutation screening of DNA can efficiently only include about 50 intron nucleotides either side of the exons, the FM-CSCE method did not find this unusual mutation in the DNA of the gene.

Dr. Abbs discussed also a new approach to preimplantation genetic diagnosis, PGD, developed in his laboratory, called *preimplantation genetic haplotyping*, PGH. Preimplantation genetic diagnosis, licensed in the UK by the Human Fertilization and Embryology Authority, HFEA, is a method whereby a single cell is removed from embryos after artificial fertilization, and tested to exclude affected embryos before implantation into the uterus.

The PGH approach to PGD entails testing the embryos for several polymorphic genetic markers in the dystrophin gene to determine which parental genes they have inherited. This one method can be applied to all suitable families, without having to develop specific tests for different mutations present in individual families. An example was

presented where 8 embryos were tested from the mother, who carried a deletion of dystrophin exons 12 to 16. The PGH test showed that 7 of the embryos had inherited the normal maternal dystrophin gene and were predicted to be 3 unaffected males and 4 unaffected females, but one female embryo had inherited the mutated X-chromosome from the carrier mother and thus would have developed to a carrier girl.

This example shows the value of PGH in enabling the parents to make certain that their next child is neither a Duchenne affected boy nor a Duchenne carrier girl. Even though the PGH test did not actually involve testing the embryos for the exons 12 to 16 deletion of the dystrophin gene, it was still essential to know the mutation in this family, otherwise the PGH test could have been looking at the wrong gene in the embryos.

Dr. Abbs finished his presentation by saying that these modern diagnostic tools are now leading to precise diagnoses for 98% of boys with clinical Duchenne symptoms and for 93% of boys with Becker symptoms. The remaining boys are likely to have other neuromuscular diseases, and in about half of these cases a mutation of the FKRP gene is found, which leads to a congenital muscular dystrophy.

Duchenne registry: *Robin Sharp* who has a Duchenne boy and is PPUK's computer specialist, described in detail the new online register which the Parent Project has established with the aim to collect all diagnostic and clinical data of as many Duchenne and Becker patients in the United Kingdom as possible. This register will have important benefits for the patients, their families, for their clinicians and other care givers, and also for the researchers who are working on the development of effective treatments.

The benefits for the patients and their families are: (1) They will have immediate access to the most up-to-date information on available management and medical procedures so that the patients can be kept in the best possible state of health. (2) The researchers will have access to the data of a large number of patients which will facilitate ongoing research projects and encourage the establishment of new projects in the interest of the patients. (3) The registry will demand that all data of a patient are recorded and thus ensure that the most modern methods are used to obtain them. (4) The inclusion of the complete molecular details of the mutation will be important for participation in clinical trials and later for the most effective therapy.

The clinicians will benefit in the following way: (1) They will have controlled access to all the data of their patients. (2) The registry will provide information on the most effective treatment for an individual patient and thus make his care optimal and patient oriented. (3) The clinician can suggest participation in the appropriate clinical trials and accompany the patient during the trial. (4) Thus, the specialized clinician or even the family doctor can maintain a central role in the care for his patient.

The researchers will also have some advantages: (1) It will be easy to recruit patients with the most adequate type of mutation for clinical trials. (2) They will also have access to a great number of patient data. (3) This will stimulate and improve research projects, and (4) make grant applications more effective.

To have a patient's data entered in the registry, the family should ask the office of the Parent Project UK to send them a form. It can also be obtained from the internet:

www.dmdregistry.org. The parents or the patient himself fill in all the information in the part that they or he can provide like name, date of birth, address, etc. The form is then given to the clinician who completes it with all the medical and diagnostic data. It is then sent back to the Parent Project office which passes it to the geneticists at Dr. *Steve Abbs*' DNA laboratory at Guy's Hospital in London, who determine the genetic details of the patient and enter all data online into the registry. The registry program then predicts treatment options like which exons should be skipped or which other genetic or pharmacological treatments are possible and available. The data forms and the predictions are returned either online or by mail to the parents and the clinician who are asked to check all entries

and to make corrections if necessary. The registry can also show the geographic location of all registered patients in the UK and thus help to recruit participants for local trials and patient support groups.

The registry has a steering committee which supervises the program's performance, suggests changes, and authorizes clinicians and researchers to have access to all or selected data. It has as its members the following geneticists and clinicians: Steve Abbs, Emma Ashton, Kate Bushby, Francesco Muntoni, Terry Partridge, Federico Roncaroli, Su Stenhouse.

All about 2,000 Duchenne and 500 Becker patients in the UK should have their data entered in the registry. And it is also open to patients in other countries.

Do not miss the wood for the trees! Final words by Francesco Muntoni.

There are very exciting developments in the field of clinical research in Duchenne muscular dystrophy. A number of very encouraging novel experimental therapeutic approaches focused on the modification of dystrophin expression (antisense oligonucleotides; PTC124) are being tried for the first time in boys with DMD and there is now realistic hope that they might have an impact on the progression of the condition. In addition other pharmacological experimental approaches aimed at making the dystrophin deficient muscle more „tolerant“ to the ongoing damage have been recently tried with success in animal models of DMD and it is likely they will enter clinical trials in the future.

In this phase of excitement it is entirely understandable

that families, patients and doctors hope that a final cure will be found soon for DMD; however it is very important to realise that the timing and the impact that these new experimental approaches will have are currently not known. This is why it is of fundamental importance to make sure that each child gets the best level of care with the various approaches that are available today to slow down the disease progression. Steroids and drugs for the heart do not „resolve“ the condition but they unequivocally allow to buy time and prolong the window of opportunity for a child to benefit from future experimental therapies. So the best way, today, to facilitate DMD boys to benefit from future therapies is that of keeping them in good shape by applying the agreed optimal standards of care.

As mentioned at the beginning of this report, I have summarized only the more scientific presentations given at the meeting in London and additional scientific information. However, there were 12 more presentations on medical and social as well as legal subjects, which, together with similar presentations at other meetings, could eventually be summarized in a special report at a later time. But it is important to draw attention to the new Parent Project working group:

PPUK Working Group on Behaviour and Learning: At the conference this year *Veronica Hinton* from Columbia University spoke in detail on her research relating to the behaviour and learning difficulties experienced by boys with Duchenne muscular dystrophy and launched PPUK's new Learning and Behaviour toolkit. *Janet Hoskin* a specialist teacher for dyslexia in London, also introduced a session on ways of assessing and implementing an early reading and spelling programme. The details of Dr. Hinton's research is now published in a new toolkit - *PPUK Learning and Behaviour Toolkit for Duchenne Muscular Dystrophy* published by PPUK. Copies are freely available from PPUK by calling *44-(0)208-556 9955.

This report, written in December 2006 and January 2007, is also available in Spanish and German. All my earlier reports in English, German, and Spanish can be seen on the internet at www.duchenne-research.com. The next annual meeting of the American *Parent Project Muscular Dystrophy*, PPMD, will take place in July 2007 in Philadelphia. This will be an opportunity to write another report on all new Duchenne research results. 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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