

Exon skipping and reading through stop codons: two research approaches for a therapy of Duchenne muscular dystrophy.

Report on a Round Table Conference in Monaco on 17 and 18 January 2004
with an interview on the time until exon skipping is ready for our boys.

Duchenne Parent Project France and the *Association Monegasque Contre les Myopathies* invited 35 scientists and representatives of Duchenne parent groups for this round-table conference in Monaco. Two scientific approaches towards a therapy of Duchenne muscular dystrophy were discussed. The following text is not a scientific report for experts but was written for the families with Duchenne boys in January and February 2004. Some of

the experiments are described in greater detail, because this report should make it clear that in spite of the incredible complexity of the human genetic structure, new experimental techniques and the skill of the scientists make it possible to manipulate the information of the very large dystrophin gene without interfering with the other about 30,000 genes in every cell of a Duchenne boy.

Introduction

Some basic genetic facts are explained
and how mutations cause Duchenne and Becker dystrophy.

Genes are functional units of the genetic material **desoxy-ribonucleic acid, DNA**. Its structure looks like an intertwined ladder, the *double helix*. The rungs of this ladder consist of four different small molecules, the **bases**: *adenine, guanine, thymine, and cytosine* (abbreviated A, G, T, C). For spatial reasons, the rungs can only contain two types of base combinations, the **base pairs** A-T and G-C. Therefore, the **sequence** of the **bases** on one strand is *complementary* to the sequence on the other,

The *letter* of the genetic information is the **nucleotide** or the **base pair**. Each of the about 100 billion (10^{12}) human body cells contains in its nucleus 46 **chromosomes** with the genetic material totalling more than six billion base pairs, grouped in about 25,000 genes.

Most of the genes carry the instructions for the construction of **proteins**. In the cell nucleus, the genetic instruction is copied, **transcribed**, from the genes to another genetic substance, the **pre-messenger ribonucleic acid** or **pre-mRNA**. Most genes consist of active regions, the **exons**, and inactive ones, the often much longer **introns**. After transcription, the introns are removed from the pre-messenger RNA, and the transcribed exons **spliced** together to the **messenger RNA, mRNA**, which then moves to the **ribosomes**, the protein synthesizing structures outside the nucleus. The ribonucleic acids, **RNAs**, use the base U, *uracil*, instead of the very similar base T of the DNA.

In the messenger RNA, three consecutive bases, a **codon** or *triplet*, signifies one amino acid according to the **genetic code**. Three of the 64 different codons, UAA, UAG, and UGA, are **stop codons**, where protein synthesis is terminated. There are no spaces between the codons. In the ribosomes, the genetic instruction of the messenger RNA is used for the construction of proteins from 20 different kinds of **amino acids**.

Duchenne muscular dystrophy is caused by a **mutation** or damage of the **dystrophin gene**. With 2.6 million base pairs, it is the longest human gene. Only 13,973 base pairs belong to the 79 exons of the gene containing the active coding sequence, the instructions for the synthesis of **dystrophin**, a long rod-shaped protein consisting of 3,685 amino acids. Dystrophin is needed for the mechanical sta-

bility of the muscle cells. It is located on the inside of the muscle cell membranes to which it is anchored by many other proteins, the **dystrophin-glycoprotein complex**.

There are three types of **mutations** or damage of the dystrophin gene: **deletions**, if one or more entire exons of the gene are missing, **duplications**, if parts of the gene are repeated, and **point mutations**, if single base pairs are exchanged, eliminated or added. As the reading mechanism in the ribosomes always reads the three letter codons one after the other without interruption, the **reading frame** is not disturbed, if the mutation deletes or adds entire codons of three base pairs each. In this case, the reading frame remains **in-frame** and the dystrophin made is longer or shorter. If this change affects non-essential structures in the central part, the *rod domains*, of the dystrophin, it can still be partly functional. Then the benign form of dystrophy, **Becker muscular dystrophy**, develops.

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

The animal mostly used for dystrophy research is the **mdx mouse**. It has a point mutation in exon 23 of its dystrophin gene that changed a CAA codon, meaning glutamic acid, to a TAA codon which is a premature stop sign. Although this mouse has no dystrophin in its muscles, its dystrophy is much less severe than that of a Duchenne boy. Therefore, experimental results obtained with these mice cannot always be expected to be the same in human patients. *A child is not a big mouse!*

Splice sites are specific sequences at the borders of exons and introns which are essential for the correct removal of the non-coding intron sequences from the pre-mRNA. The splicing itself is accomplished by **spliceosomes**, a complex of many small RNAs and proteins whose details and interplay are still not completely understood.

Oligonucleotides – oligo means few – are short nucleic acids composed of, e.g. 15 to 30, nucleotides. They can be

oligoribonucleotides or oligodesoxyribonucleotides depending on whether they have an RNA or a DNA structure. For exon skipping they bind to the targeted genetic sequences of the pre-mRNA according to the specific base pairing rule characteristic of all nucleic acids: A binds to U or T, and G to C, and vice versa. For a correct binding, their sequence must be “**antisense**”, meaning that the sequence must be reversed in relation to the sequence to be targeted. The reason is that nucleic acids have polarity, a direction from the so-called 5’ to the 3’ ends. The example at the end of this report explains these relationships.

There is still no cure for Duchenne dystrophy. *Jean-Claude Kaplan* (Paris) said in his introductory address that he remembered a conference in Versailles on Duchenne research in 1987 when everybody was so excited after the detection one year before of the gene whose mutations cause Duchenne dystrophy and of its product dystrophin, believing that a therapy seemed to be close. Now, 17 years later, there is still no cure. For the researchers who now see the severe scientific problems, that conference seems to have taken place yesterday. But for parents it is quite a different story, 17 years is a generation of Duchenne boys!

But research has opened many avenues, although Duchenne dystrophy is not the easiest disease to be treated by

gene manipulation. Several techniques now exist for transporting the active parts of the gene into muscle cells or shorter versions of it, to repair its mutations, to ignore the stop signs that appear in the wrong places, and to try to influence the progress of the disease with drug-like substances. But, with the exception of steroids, all this has *not* been done in living boys, but in their isolated muscle cells in the laboratory or in animals. However, clinical trials are being planned now for the near future, mainly trials with the – at present – most promising approaches, exon skipping and stop codon read-through.

Nevertheless, when they have been fully developed these two techniques will only be able to help those Duchenne boys whose exact mutations are known. And it should also be known which symptoms the presence of an altered dystrophin, shortened by exon skipping, would cause. In many cases, this can be predicted, but there are exceptions to the reading frame rules. Therefore, large data banks with detailed genetic and clinical information on individual patients will be important. Such data banks already exist or are being established at the Cochin hospital in Paris, at the universities of Utah in Salt Lake City and in Leiden in the Netherlands. Their information is freely available to all researchers.

Exon skipping can change a Duchenne into a Becker mutation.

Short artificial oligonucleotides can be injected into mouse and human Duchenne muscle cells in culture and into living mice to induce the synthesis of shortened dystrophin.

These oligonucleotides can also be made in muscle cells which have received their genes.

Clinical trials with Duchenne boys are being prepared, and one has already been done in Japan.

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

Exons can be eliminated from the mRNA with *antisense oligoribonucleotides*. They are short RNA structures consisting of about 20 nucleotides whose sequences are constructed in such a way that they attach themselves only at the corresponding – complementary – sequence inside the exon to be removed or at its borders *and nowhere else*. These oligonucleotides thus interfere with the splicing machinery so that the targeted exons are no longer included in the mRNA, they are *skipped*.

The gene itself with its mutation is not altered by *exon skipping*, but its mRNA no longer contains the information of the skipped exon. As the mRNA is shorter than normal, the dystrophin protein is also shorter, it contains fewer amino acids. If the missing amino acids are part of the central region of the dystrophin, they are often not essential, and the resulting shorter protein can 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.

This technique will influence the conversion of pre-mRNA into mRNA. As the dystrophin gene itself will *not* be altered, the effect of the antisense treatment will not be permanent, so it will have to be repeated at intervals still to

be determined.

As the sequence of the base pairs of the entire dystrophin gene including the borders of all its 79 exons are known in detail, one can predict which exon or exons would have to be eliminated – *skipped* – to restore the reading frame of an individual patient. However, it is not certain whether the experimental results now being obtained in cell culture or in mice, will be the same in Duchenne boys, and whether the shortened dystrophin will really lead to the clinical symptoms of a Becker muscular dystrophy. At this time, such predictions can only be purely theoretical.

The splicing process can be changed in laboratory experiments. *George Dickson* (London) discussed the different methods to induce exon skipping, i.e., by interfering either with the spliceosomes at one or both ends of the exons or by blocking sequences entirely inside the exon sequences. Antisense oligonucleotides – in most cases RNA structures – are unstable molecules. To increase their lifetime, they have to be modified. Adding one carbon and three hydrogen atoms to the sugar units of the RNA and also to the cytosine bases, as well as exchanging one oxygen atom for a sulfur atom in the phosphoric acid part of the structure, stabilizes them. And even the entire ribose units can be exchanged for amino acids, and these peptide nucleic acids, PNA, are sometimes also quite active.

The experiments were mostly done in *cultured muscle cells* from mdx mice whose point mutation in exon 23, causing a premature stop codon, can be avoided by elimi-

nating this entire exon from the mRNA by skipping it during the splicing process. As the ends of that exon are between the codons but not inside them, its loss does not disturb the reading frame, but it makes the dystrophin slightly shorter.

To find the most effective oligonucleotides, their base sequence was changed so that their attachment sites could be moved across the exon-intron borders. Oligonucleotides of different length were also used. The optimal size seems to be 18 nucleotides and the best attachment site located at the 5' end of the exon. Up to 60% of the dystrophin mRNA had the sequence of exon 23 missing. In these laboratory experiments, it was shown that this led to the production of new dystrophin and to its correct localization underneath the cell membrane.

In the discussion, it was said that there are two ways to do exon skipping: either to target – to block with the oligonucleotide – the internal sequences of the exon or to target the sides of the exon. But it remains unclear which approach would be more specific, i.e., leading to skipping of only the exon intended and not of others: targeting sequences in the introns at the border to the exons which show some similarities, or targeting sequences entirely within the exons which may be the same in the genes of similar proteins like, e.g., utrophin, because they were conserved by evolution.

Exon skipping in isolated muscle cells from patients shows that Duchenne mutations of most patients can be corrected:

Judith van Deutekom (Leiden) said that exon 45 of the dystrophin gene is the single most frequently deleted exon in Duchenne boys causing an out-of-frame mRNA. However, if both exons 45 and 46 are missing *simultaneously*, the reading frame is not disturbed. In laboratory experiments with a culture of muscle cells from a Duchenne patient with an exon-45 deletion, exon 46 could be skipped. The dystrophin produced was shorter than normal, 108 non-essential amino acids from the central part of the protein were missing. Patients with this 45-46 double deletion are known to have the milder symptoms of Becker dystrophy.

The skipping of exon 46 in these human muscle cells was caused by an *antisense oligoribonucleotide* that attached itself to a specific sequence *within* exon 46. After 16 hours, new dystrophin could be detected in the cells, and after 48 hours it had moved to the cell membrane. With the same technique, it was possible to skip 19 other exons in cell cultures of human muscles. Not only deletions that cause Duchenne dystrophy can be corrected with this technique, but also point mutations. In these cases, the exon containing the point mutation has to be skipped and, in addition, one of the neighboring exons that has the correct borders to restore the reading frame.

As some out-of-frame mutations cannot be corrected by skipping only one exon, more than one exon could be skipped by applying the specific oligonucleotides for all exons to be skipped at the same time. This *double and multiple exon skipping* could even be made more effective by connecting the oligonucleotides chemically so that practically no single-exon skipping occurred when more exons had to be skipped simultaneously. And by targeting exons 45 and 51 in the deletion hot spot of the gene at the same time, all exons between 45 and 51, i.e. the exons 46 –

50, were also skipped regardless of whether they were deleted by the mutation or not. Thus, with this one set of two connected oligonucleotides, 17% of all Duchenne boys with deletions could be treated.

In human cell cultures, up to about 80% of the targeted exons could be skipped in this way. Some of the oligonucleotides added were still present after a month, thus they can cause skipping for a longer time. And that means that these potential drugs will possibly have to be applied not daily but in rather long time intervals.

Single and multi-exon skipping would theoretically be able to change Duchenne into Becker dystrophy in *more than 90%* of all patients with deletions, duplications, and point mutations. Less than 10% of Duchenne mutations will not be treatable, namely those that destroy essential regions at both ends of the dystrophin, its promoter sequences, or which lead to large scale rearrangements of the dystrophin gene.

Exon skipping also works in living mice: *Terry Partridge* (London) reported that the in-vitro technique described by George Dickson to skip exon 23 in the dystrophin mRNA of mdx mice was also successfully used in living animals. The protected antisense oligoribonucleotide was prepared in Steve Wilton's laboratory. It consisted of 20 nucleotides whose sequence was complementary to one of the border regions of exon 23. About 5 micrograms (millionths of a gram) of this stabilized potential "gene drug" were injected into a small leg muscle of two-week old mice together with the polymer F127 which helps the oligonucleotide to enter the cells. After two to four weeks, up to 20% of the muscle fibers contained almost the normal amount of slightly shortened dystrophin. And, together with the other components of the dystrophin complex, it was located correctly on the cell membrane. Muscle force was significantly improved but not completely normalized. A repeated treatment increased the number of dystrophin-positive muscle fibers without the development of an immune rejection.

In the most recent experiment, a systemic approach, which would reach all muscles, was attempted. Two milligrams of the oligonucleotide was injected together with F127 into the veins of mdx mice. Preliminary results show that small amounts of new dystrophin could be detected at different levels in many muscles. There is no explanation yet for this variation.

Which is the best oligonucleotide? *Steve Wilton* (Perth) reported on experiments with the aim to find the best modification of the basic structure of the antisense oligonucleotides so that they meet a number of requirements: crossing the membranes of the muscle cells and their nuclei easily; inducing specific exon skipping at high rates with low concentrations; being sufficiently stable, resistant against degradation, and without side effects that could be deleterious.

The following modifications of the oligonucleotides are possible, many have been realized and their effects analyzed in cell culture experiments: (a) exchanging one non-bridging oxygen atom of the phosphoric acid backbone for a sulfur atom; (b) protecting each ribose unit with a methyl group of one carbon and three hydrogen atoms; (c) adding methyl groups to some of the bases; (d) adding cholesterol,

four connected carbon rings with a tail, to the ends of the oligonucleotide structures; (e) retaining a bulky trityl protecting group, three benzene rings connected by one carbon atom, to the 5' end; (f) examining the morpholino chemistry and enhancing its delivery by adding a "leash" of another oligonucleotide to allow "lipoplex" formation.

One of the best performing antisense oligoribonucleotides was used in the mouse studies in *Terry Partridge's* laboratory. It is targeted to the splice site at the border between exon 23 and intron 23 of the mouse pre-mRNA. It is 20 nucleotides long and has the sequence GGCCAA-CCUCGGCUUACCU. All the 2'OH groups of the ribose units are protected with methyl groups and one oxygen atom of its phosphate groups is exchanged for a sulfur atom at each backbone position.

Preparation of a clinical application of exon skipping:

Masafumi Matsuo (Kobe) reported on his experiments which were mainly undertaken to correct the deletion of exon 20 in a Duchenne patient. The disturbed reading frame after this deletion can be corrected by skipping exon 19. The researchers experimented with a series of *antisense oligonucleotides*, whose sequence enabled them to attach themselves to and thus to block the *exon splicing enhancer* sequence *within* exon 19 that is necessary for the normal inclusion of this exon in the mRNA. These oligonucleotides were short chains of DNA structure. They were stabilized by substituting a sulfur for an oxygen atom of their phosphate groups. One of these antisense oligo-desoxyribonucleotides, GCCTGAGCTGATCTGCTGGC-ATCTTGACAGTT, 31 nucleotides long, was especially effective in skipping exon 19 in cultures of developing muscle cells from the Duchenne patient with an exon-20 deletion. After the skipping, new dystrophin that was about 3% shorter than normal human dystrophin could be detected in up to 90% of the cells. Other specific oligonucleotides were constructed which could effectively skip exons 41, 44, 45, 46, 51, 53, and 55 which would repair frequent Duchenne mutations in one of the deletion hot spots of the dystrophin gene.

Further laboratory experiments for the preparation of clinical trials with Duchenne boys: *Gert-Jan van Ommen* (Leiden) outlined the experiments to be performed before oligonucleotides can be injected into children.

Transgenic mice were created by genetic techniques which have, in addition to their own normal dystrophin mouse gene, the entire human dystrophin gene in all of their muscle cells. The presence of the human gene in these non-dystrophic mice led to the production of normal *human* dystrophin which is located together with the slightly different normal mouse dystrophin under the cell membranes of the mouse muscles correctly anchored there by the proteins of the dystrophin complex. When these mice are crossed with dystrophic mdx-mice without dystrophin, animals were obtained which only have the human dystrophin in their muscles. And these *humanized mdx-mice* are not dystrophic any more!

Antisense oligonucleotides against sequences *within* the human exons 44 and 49 were injected into the normal mice with human dystrophin with the result that only the human dystrophin had the amino acids missing which are specified by these two exons, whereas the slightly different

mouse dystrophin was left unchanged. These experiments showed that exon skipping with this technique is very specific, it eliminates only the targeted exons of the dystrophin mRNA, and probably does not interfere with the other about 30,000 human genes.

With new experiments, the Dutch researchers will try to introduce the deletions found in Duchenne patients into the human gene inside the humanized mdx-mice. This will allow them to determine whether the specific oligonucleotides prepared for Duchenne boys really skip exons in a living organism.

If these preparatory experiments show the expected results, the first clinical trial with Duchenne boys will follow, probably in the second half of 2004. It will have to be approved by a regulatory agency and an ethical committee of the university. The antisense oligonucleotides will be manufactured in pure clinical quality by the *ProSensa* company in Leiden under the supervision of *Gerard Platenburg*. They will be tested chemically and in mice. ProSensa will organize and manage the trial and will also be responsible for following all safety requirements. Injections and the clinical follow-up of the children will be done in the university hospitals in Groningen, Amsterdam, and Louvain.

For the clinical trials, the oligonucleotides will have to be modified chemically like the ones used in the experiments in *Terry Partridge's* laboratory. Then they can cross the cell membrane more easily and are not destroyed prematurely by cell-protecting enzymes.

When the protocol is completed, submitted and agreed by the regulatory authorities, a few Duchenne boys between ages 12 and 16 years will participate in the first trial. In the phase-I trial, the oligonucleotides will be injected into the tibialis anterior muscle. This small muscle, which lifts the foot, is not much affected in Duchenne children of this age.

Exon skipping with antisense oligoribonucleotides would be a completely novel type of medication. These substances are not conventional drugs, but they will interfere with the activity and the regulation of a human gene. In addition to the predicted effects, they might have entirely unforeseen and possibly negative consequences. The legal and regulatory authorities have not yet been confronted with such a new therapeutic approach and their reaction is unpredictable.

Exons can be skipped by altering the sequence of the gene itself: In *Thomas Rando's* (Stanford) laboratory a *gene editing* approach was used to eliminate mouse exon 23 from the mRNA to restore the disturbed reading frame. This technique would change the sequence of the gene itself, thus leading to a permanent alteration that would avoid the need for repeated applications of oligonucleotides.

For this approach, an attempt was made to change a single base pair at the splice site sequences of exon 23 by using RNA-DNA double-stranded oligonucleotides, so-called *chimeric* structures. Their sequences were not exactly complementary to the splice site region but contained one error, one mismatched nucleotide. All cells have a group of enzymes that repair sequence errors which appear rather infrequently when the genetic material is duplicated during cell division. This repair mechanism would use the

incorrect instruction of the chimeric oligonucleotide to introduce a base pair change at the splice site. This deliberate "error" would destroy the normal splicing reaction at this site with the result that this particular exon is skipped, not included any more in the mRNA.

In laboratory experiments with cell cultures from mdx mouse muscles, chimeric oligonucleotides directed to the splice sites around exon 23 were added to the culture medium. Although 2 to 5 % of the cells contained dystrophin afterwards, the new dystrophin was not homogeneous but consisted of up to 5 shorter versions. This meant that this technique did not only cause the intentional removal of exon 23 from the mRNA but also the uncontrolled skipping of many different exons around exon 23.

These experiments proved that permanent single base-pair changes can be made in the dystrophin gene. However, this is an evolving research field, and there are still many problems to solve before this chimera-technique could contribute to a Duchenne therapy.

Exons can be skipped with oligonucleotides produced inside mouse muscle cells: *Daniel Schümperli* (Berne) discussed a new method of skipping exons in which the antisense oligonucleotides do not have to be injected but are synthesized in the cell nucleus after transfer of their genes.

The intron sequences are cut out from the pre-mRNA in the cell nucleus by spliceosomes. They are complex structures consisting of several proteins and small RNAs, small nuclear = snRNAs, which recognize the exon-intron borders and then connect the exons after splicing precisely and without shifting the reading frame. Some of these very short RNAs are the U7-snRNAs. They normally regulate cleavage reactions involved in maturing the mRNAs of histones which are proteins necessary for packaging the DNA in the chromosomes.

For this new method, the U7-snRNAs were genetically modified so that they no longer bound to the histone pre-mRNA but only to the splice sites in the region of exon 23 of the pre-mRNA of the mouse dystrophin. To achieve this, a gene coding for the modified U7-snRNA together with a creatine kinase enhancer was packed into plasmids as vectors and then transferred into isolated myoblasts of the mouse. These cells then developed in the culture dish to muscle cells.

In the nuclei of the treated myoblasts, the transferred gene produced modified U7-snRNAs. They had new recognition sequences and now bound in front and behind exon 23 of the dystrophin pre-mRNA to sites important for the splicing process. Thus, these splice sites were blocked and exon 23 was removed together with the introns during the splicing of the pre-mRNA. As the point mutation of the mdx mice is localized in exon 23, the removal of this exon enabled the mdx muscle cells, which could not make any normal dystrophin, to produce a slightly shortened dystrophin protein which then moved to its correct place underneath the cell membrane.

The aim of this fundamental gene experiment outside of the living mouse was to prove that muscle cells themselves can produce therapeutic antisense oligonucleotides. This method would lead to a continuous production of the therapeutic compound which would make it unnecessary to apply them repeatedly. For human application, U7-

snRNAs adapted to the individual mutation of a Duchenne patient would have to be made, whose gene must then be transported with a gene therapeutic vector into the cell nuclei of the muscle fibers. An alternative would be a transfer of the U7-snRNA genes into satellite cells or other muscle stem cells outside the body and then injecting them into the blood stream or directly into the muscles. As the U7-snRNA genes are very short, this transfer would probably be easier to perform than the transfer of the coding DNA sequences for the entire or the shortened dystrophin as is being tried in other approaches. Moreover, since no dystrophin is made outside of muscle cells, this approach should not elicit adverse toxic or immune responses.

Human muscle cells can also produce oligonucleotides for skipping exons: *Irene Bozzoni* (Rome) extended the experiments described by *Daniel Schümperli* to muscle cells from human patients. To this end, three kinds of the snRNAs in the spliceosomes were modified to induce the skipping of exon 51 in muscle cell cultures from a patient with a deletion of exons 48 to 50.

In the spliceosomes components U1-snRNA, U2-snRNA, and in two preparations of the U7-snRNAs, some nucleotide sequences were exchanged for specially designed artificial sequences with the following consequences: the modified *U1-snRNA* recognizes and blocks the splice site at the border region of exon 51 and the following intron. The modified *U2-snRNA* attaches to the sequence containing the branch point in the intron that the spliceosomes need for splicing the neighboring exon. And it no longer binds to the U6-snRNAs which would activate splicing at this site. One preparation of the modified *U7-snRNAs* loses its normal activity in the synthesis of the histone proteins and interacts now with the splice site at the other end of exon 51 blocking the splicing mechanism there. The other *U7-snRNA* preparation, the *double construct*, contains two antisense sequences against the splice sites at both ends of exon 51 and thus can fold the exon sequence to be skipped into a tight loop.

As all these modified snRNAs need to be produced continuously in muscle cell nuclei, their correspondingly modified genes with their own promoters were transported into the human muscle cells in culture by a recombinant retrovirus preparation as gene vector. It could be shown that, after the gene transfer, the treated muscle cells made the different modified snRNAs at fairly good levels. But only one of the three snRNAs targeted to a single splice site, the modified U1-snRNA, induced the intended skipping of exon 51 and produced the correct dystrophin mRNA without the exons 48 to 51 at about 10% of the normal level.

But with the U7 double construct, which reacts with both ends of exon 51 simultaneously, *more than 60%* of the new mRNA without exons 48 to 51 was produced. And this led to the production of the shorter-than-normal dystrophin lacking the 210 amino acids coded for by the base sequences of the deleted exons 48 to 50 and the skipped exon 51. In similar experiments, exon 45 was skipped in muscle cell cultures from a patient with a deletion of exons 46 to 51.

The Italian researchers are now planning a cooperation with the research team of *Giulio Cossu* in Milan which has shown that *mesoangioblasts* from the cell wall of fetal

blood vessels are stem cells. They can be grown extensively in culture and, when injected into the blood circulation of living mdx mice can migrate into dystrophic muscle cells across their damaged cell membranes.

It is now planned to modify mesoangioblasts from mdx mice so that they can produce the antisense snRNAs against the splice sites of exons. They will then be re-injected into the mdx mice. This technique would avoid the use of viruses as gene vectors and thus make this technique a promising therapeutic approach to change Duchenne dystrophy of the majority of patients into Becker muscular dystrophy permanently .

Viruses can get the snRNA genes into muscle cells: *Luis Garcia* (Paris) presented his work with adeno-associated viruses which were used as vectors to transport the modified genes for the modified snRNAs of the spliceosomes into the nuclei of muscle cells. Muscle researchers now have great experience with these small viruses because they are also widely used for gene-therapy experiments with shortened dystrophin genes.

The rather small genes for the modified U7-snRNAs as prepared by *Irene Bozzoni* and *Daniel Schimperli* for the skipping of exon 23 in mice and of exon 51 in human muscle cells were packaged into these small viruses, and then applied to muscle cell cultures or injected into living mice.

For a future Duchenne therapy, the therapeutic agents will have to be applied systemically via the blood circulation. Therefore, in a preliminary experiment, one milliliter of a solution containing 100 billion (10^{11}) virus particles carrying the gene for the U7 double construct were injected within one minute into the arteries of mice while the corresponding veins were temporarily blocked by clamping. This injection under pressure enhanced the passage of the viruses through the cell membranes and led to the appearance of new dystrophin in many different muscles. In some instances, 90% of the fibers were dystrophin-positive. By injecting these recombinant viruses into the blood circulation, they not only enter the muscles but practically all other organs, too. But no skipping in other genes seem to occur, probably because the target sequences of the modified U7-snRNA are only present in the dystrophin pre-mRNA and nowhere else.

The same technique also skipped exon 51 in human muscle fibers, but not at the same efficiency as found by applying the U7-snRNA with the two attachment sites. Work continues to find the optimal conditions for this procedure.

Exon 19 was skipped in a Duchenne patient, the first clinical trial: In his second presentation, *Masafumi Matsuo* (Kobe) reported on the extension of his laboratory experiments described above to the treatment of a Duchenne patient with the exon-skipping technique.

For further preparations of the first clinical application of this technique, an oligonucleotide with a single-strand DNA structure for exon-19 skipping was used. It could be

shown that its addition to myocytes from a biopsy of the patient with the exon-20 deletion could skip exon 19 so that, after 10 days, the 3% shorter-than-normal dystrophin could be detected in about 20% of these muscle precursor cells of the patient.

The next step was to show that this technique was also effective in living mdx mice. The absence of dystrophin makes their muscle cell membranes permeable to potential drugs. The application of 2 mg (milligrams) of the oligonucleotide per kg body weight by injection into the abdomen led indeed to the intended exon skipping. After two days, messenger RNA without exon 19, but still with its mdx point mutation in exon 23, could be detected not only in skeletal but also in cardiac muscle and persisted there until at least 14 days after treatment. To check for possible toxicity, mice were injected with one hundred times the intended dose for the patient, i.e. 50 mg per kg body weight. No toxic effects could be detected.

After these successful in-vivo experiments, the responsible ethical committee gave its permission to treat a 10-year old wheelchair-bound Duchenne boy with the exon-20 deletion, whose muscle cells had been used for the preparatory experiments. He received four infusions intravenously, into his blood circulation, once a week for four weeks. 0.5 mg per kg of body weight of the antisense oligodesoxyribonucleotide dissolved in saline (salt water) without a carrier substance were infused during two hours.

One day after each infusion, the dystrophin mRNA was analyzed in the lymphocytes, one kind of white blood cells. After the second infusion, new mRNA could be detected whose amount increased with each following infusion. Because this mRNA no longer contained the 88 nucleotides of exon 19, it was smaller by 0.64% than the still present normally deleted RNA. One week after the last infusion, a sequence determination showed that the end of exon 18 was joined directly to the beginning of exon 21 in the mRNA. Thus, not only exon 20 was missing, which caused the Duchenne dystrophy, but exon 19 was not present either. Finally, in tests with antibodies, new dystrophin could be detected in the muscle cells after another biopsy. Additional and more detailed protein analyses will be made in the near future.

An improvement of the boy's muscle function could not be detected yet. The CK activities were not changed significantly. The transfused oligonucleotide was apparently sufficiently stable to be detectable several weeks after the application. The patient is now being closely supervised and clinically investigated. It is planned to repeat the treatment with four consecutive infusions after one year. It may also be decided to repeat single infusions in shorter intervals like once or twice per month.

If this first systemic exon-skipping treatment turns out to be really as successful as it looks at present, skipping of exon 19 could also be tried in Duchenne boys with other deletions, like exons 8-18, 20-22, 20-23 and more extensive deletions starting with exon 20 and ending with exon 24 and all following exons up to deletion 20-42.

Reading through stop codons.

**The molecular basis for this treatment with gentamicin is explained.
The conditions are examined and a better potential drug is presented.
The results of the first clinical trials are reported.**

Ignoring a premature stop codon by antibiotics: *Jean-Pierre Rousset* (Paris) described in detail how the biosynthesis of proteins is terminated at normal stop codons in the mRNA, what happens when a premature stop codon is caused by a point mutation, and how an antibiotic like *gentamicin* can make the synthesis mechanism ignore this premature stop sign by *reading through* it.

The many kinds of proteins a cell needs for its function are synthesized in ribosomes, large complex structures consisting of four large RNAs with enzymatic activity, *ribozymes*, and about 80 different proteins. The genetic information for the construction of proteins is brought to the ribosomes by mRNAs. Inside the ribosomes, the new protein is assembled from its building blocks, the amino acids. They are delivered to the assembly site by another kind of RNAs, the transfer or tRNAs, which recognize the triplet codons of the mRNA one after the other. When a normal stop codon arrives at the synthesis site, meaning that the protein is ready, there is no tRNA which would bring an amino acid, but special proteins, *release factors*, enter the assembly site, bring the synthesis to a halt and release the complete new protein from the ribosome.

About 5% to 10% of Duchenne boys have a point mutation in their dystrophin gene which changed an amino acid code word 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 ready. Quite likely, mRNAs with such premature stop codons will be degraded via the *nonsense mediated decay* pathway, and the unfinished dystrophin will also be rapidly degraded.

Gentamicin is an antibiotic that causes the RNA translation mechanism in the ribosomes to ignore such a premature stop codon, i.e. to *read through* it. Read-through efficiency is different for the three different stop codons and they also depend on the mRNA sequence before and after the stop sign. And there are different kinds of gentamicin with slightly different structures, this influences also the extent of read-through. The details of these complex relationships are still not completely understood.

If the read-through technique, which now shows a wide variation of efficiency with different mutations from patients, can be developed to a Duchenne therapy, only a minority of all patients, about 5% to 10%, will probably be able to benefit from it. The new dystrophin will practically have the normal structure. Therefore, on condition that sufficient new dystrophin is made, this therapy will cure the disease and not only change it to a Becker-type dystrophy. As read-through does not occur at the gene level but during protein synthesis in the ribosomes, treatment will have to be repeated periodically. Patients with a point mutation should have the sequence around the mutation site determined, so that the efficiency of a treatment can be predicted. Gentamicin has the advantage of being a well known drug whose alternative use as a possible therapy for Duchenne muscular dystrophy would not require long

approval procedures.

Gentamicin can cure muscular dystrophy in mdx mice:

Lee Sweeney (Philadelphia) discussed the effect of gentamicin in mdx mice. These dystrophic mice have a point mutation that exchanged the base cytosine (C) in position 3,185 in exon 23 against a thymine (T). This converted the CAA codon, which normally codes for the amino acid glutamine, to the triplet TAA which is a premature stop codon. Gentamicin injections into mdx mice led to the appearance of new dystrophin in up to 20% of the muscle fibers. And mice with this amount of dystrophin had ameliorated clinical symptoms. But gentamicin retards the growth of the animals and in humans can cause serious side effects like inner ear and kidney damage.

As the researchers had difficulties when repeating the experiments, they analyzed the different commercial batches of the drug and discovered that gentamicin exists in 5 varieties with slightly different structures. Although they all had the same antibiotic activity, their read-through efficiency was quite distinct and their toxicity also. The C2 component had the highest read-through efficiency and the lowest toxicity. This means that gentamicin preparations would have to be carefully selected before they are used for clinical trials.

The sequence in the region around the stop codon influences the read-through efficiency:

Kevin Flanigan (Salt Lake City) reported on the development of a test system to determine in what way a stop codon and the following nucleotide influences the efficiency of the read-through reaction with antibiotics. For this system, DNA oligonucleotides of 21 units length were used. Each of these short sequences contained one of the four stop codons TGA, TAG, or TAA, and each of these were followed by one of the four DNA nucleotides, A, G, C, or T. Every one of these 12 DNA sequences were then connected to the genes for two light producing enzymes and transported with a virus into a culture of myotubes, muscle precursor cells. One of the enzymes produces light only when the oligonucleotide construction has entered the myotubes, and the other produces light only when the stop codon did *not stop* the production of the enzyme's mRNA. Thus, this system allows to determine the read-through efficiency of gentamicin and other antibiotics by simply measuring the intensity of the light produced by the two reporter enzymes.

In this way, it could be shown that the RNA stop codon UGA allowed a higher read-through than the two other codons UAG and UAA, and that the base following the codon was important, too: C led to a higher read-through efficiency than U; and A and G were still less effective. The most active combination was UGA-C, it allowed a read-through in the presence of gentamicin of 8% in the test system. The UAA-A stop codon of the mdx mice only gave an 1%-read-through efficiency. Other aminoglycoside antibiotics were also tested with this system: geneticin

was more effective than gentamicin, but it is more toxic. Amikacin, tobramycin, and paromomycin were less active.

In this artificial test system, the 18 nucleotides in front and behind the stop codon had a random sequence, as for instance TCG-ACG-TGC-GAT-TGA-CCG-TTC with the stop codon and its following nucleotide underlined.

As it is not yet possible to predict the read-through efficiency from the sequence around the mutation site of an individual patient, it will be advisable to determine the probable read-through efficiency of the gentamicin preparation in a laboratory experiment *before the treatment* of the boy. The new test would allow this if the sequence of the oligonucleotide in the test system is identical to the sequence of the patient's mutation-site region.

In *Kevin Flanigan's* laboratory, a new analysis method – SCAIP – was developed for rapidly sequencing all active parts – the exons, promoters, and splice sites – of the entire dystrophin gene. This method is now used to prepare data banks which will allow the clinical course of a Duchenne or Becker muscular dystrophy to be predicted based on the precise knowledge of the patient's mutation. This new method is also ideally suited to finding those 5% to 10% of patients who would possibly benefit from a read-through therapy.

A new chemical compound that is better than gentamicin: The company *PTC Therapeutics* in South Plainfield, New Jersey, is developing small molecule drugs to interfere with mutated RNA structures that are responsible for hereditary diseases. Finding a therapy for Duchenne muscular dystrophy, based on stop codon read-through is one of its priorities. *Lee Sweeney* (Philadelphia) is one of their scientific advisors. He gave a summary of the experimental results so far. (PTC means premature termination codon.)

With a test system similar to the one described by *Kevin Flanigan*, almost one million chemical compounds were automatically tested for their ability to cause read-through of premature stop codons. The most promising compound identified is PTC124 whose chemical structure is still kept confidential. It is about 50 times more efficient than gentamicin in restoring the dystrophin synthesis in mdx mice, and it can be given *orally*, a very important advantage for a future therapy.

No read-through of normal stop codons nor exon skipping nor alternate splicing were detected. Only full-length dystrophin appeared in almost normal amounts in cell culture or in 20% of the muscle fibers of mice and dogs after oral application. PTC124 disappears from the animals much faster than gentamicin. For a long-term human therapy it will probably have to be given two or three times a day. Toxicity studies in mice and dogs with 50 times the probable therapeutic concentration in humans did not show any side effects.

A clinical phase-I trial will begin, not with patients but with healthy volunteers in the second half of 2004. A phase-II trial will follow immediately. The drug is probably also active for premature stop codons in the gene responsible for cystic fibrosis.

The first clinical trials were disappointing: *Kenneth Fischbeck* (Bethesda) reported on a clinical trial with four patients which was performed 3 years ago at the National Institutes of Health in Bethesda near Washington. Two Duchenne patients, 13 and 16 years old with stop codons UAA and UAG, and 2 Becker patients, 6 and 18 years old, both with a UGA stop codon, participated in this first clinical trial. They received 7.5 mg per kg gentamicin intravenously over one hour per day for 14 days.

After treatment, *no* new full-length dystrophin could be detected although one of the Becker patients had a C following the stop codon UGA, a combination which showed the highest read-through activity in experiments with mice and the artificial test system. There was no evidence of toxicity and no change of muscle strength, The creatine kinase activities decreased significantly during treatment but increased again afterwards.

The trial was performed before it was known that gentamicin preparations are mixtures of different components with varying read-through efficiencies. It was concluded that future trials should be over longer periods and only with gentamicin preparations of proven high efficiencies.

A similar trial with 12 patients was performed in Columbus/Ohio by *Jerry Mendell*. The results were equally disappointing. A new larger trial is now being prepared.

Another clinical trial was more successful: *Luisa Politano* (Naples) presented the results of a similar clinical trial in Naples with four Duchenne patients, one 4 years old with a UGA-C stop codon in exon 29, two brothers, 8 and 10 years old with an UGA-C stop in exon 7, and an 11 year old patient with a UAA-A stop codon in exon 6.

The main constituent of the commercial gentamicin preparation was the most active C2 component. The drug was administered intravenously over two hours daily for 6 days at a dose of 6 mg/kg dissolved in saline. Then the patients received no medication for 7 weeks, and as no side effects were observed, treatment was repeated for another 6 days at the slightly higher dose of 7.5 mg/kg.

Before and after the treatment, open biopsies were performed for the determination of dystrophin. In the 4-year old patient, apparently normal dystrophin could be found after treatment, however, the results of tests with antibodies left some questions open. In two of the other patients, a number of shorter dystrophins were detected. As in the two trials in the United States, the CK activities decreased during the treatment but afterwards increased again

Treatment of the 4-year old patient was continued with 12 more cycles of 6-day applications of gentamicin without any side effects. He is now 7 years old and has no Duchenne symptoms any more.

In the discussion it was argued whether the new full-length dystrophin and the shorter versions were due to stop codon read-through or possibly to alternate splicing reactions or even to non-specific exon skipping. And the mutation in exon 29 may have created a stop codon with a special structure, so that this positive result may be an exceptional case that cannot be generalized.

In the general discussion, the following points were raised:

On exon skipping: Targeting the oligonucleotides to sequences entirely within the exon to be skipped seems to be more specific than targeting the splice sites at the exon's borders. --- The kind of chemical modification of the oligonucleotides also seems to influence skipping. --- Experiments in cell culture and in animals or humans might give different results. --- In patients who have the same mutation, the exon skipping results may be different. One must find his own optimal oligonucleotide for each patient --- Nobody knows how exon skipping really works.

Before a clinical trial with patients is started, it would be important to try the oligonucleotides on healthy volunteers. However, an unintended skipping of an exon, e.g. of exon 51 that shifts the reading frame, may give muscular dystrophy to a healthy person. ---- Even if there is often no immune response in mdx mice, this might be different in children. Before a clinical trial, it should be checked whether an immune reaction against new dystrophin is likely or not.

It is not possible to predict reliably whether a dystrophin shortened by exon skipping really causes a Becker dystrophy. The effect of some deleted dystrophins are known, e.g., the deletion of exons 45 to 47 creates a very mild Becker dystrophy. But others will just have to be tried in patients. Although the experiments could be done with the "humanized" mice, the mouse is not a good clinical model of the human disease. And it is very difficult, expensive and time consuming to make all the specific transgenic mice. --- The skipping reaction might also depend on still unknown controlling factors. A micro array analysis before and after treatment might reveal unexpected changes in other proteins. But this creates huge amounts of data which regulating committees do not understand and the scientists often don't either. --- It is not yet known whether dystrophin that appears after exon skipping has the same three-dimensional structure as "normal" Becker dystrophin due to a mutation. --- It is also not known whether the amount of dystrophin produced after skipping is sufficient to alleviate the symptoms of the disease. --- The deletion break points in the gene can be somewhere inside the introns. This may have consequences for the clinical course of the disease. But no attempts have been made to identify the break points precisely in the often very long intron sequences.

Toxicity effects must also be looked for. A clinical phase-I trial normally detects toxic effects. They might be due to unintended exon skipping in other genes.

The first clinical trials will use local intramuscular injections of oligonucleotides. Then, toxicity, immune problems and other side effects can be seen much better than after a systemic intravenous injection. --- The next step to systemic application is quite big. It should only be done if one is certain that it will work, e.g., by injecting an oligonucleotide locally in one muscle of the patient for whom it was made. This would take only a few weeks and would make the procedure safer. If anything happens, one could interrupt the experiment. If anything happens during a systemic injection, the drug would go everywhere in the body and may create another disease like myositis on top of muscular dystrophy. We do not know how patients will

react to their drugs, we do not want any disasters!

Clinical phase-III trials to determine the optimal dosage must be done with a great number of patients. As there will not be enough Duchenne patients for such a large trial, a therapeutic method will then remain an experimental therapy after a phase-II trial. Hopefully, such an effective drug will be approved as part of an orphan disease legislation. -- - There are examples in gene therapy for cancer where regulating agencies do not insist on following all the rules.

Many Becker patients have quite weak clinical symptoms, and it is expected that there are many people with short dystrophins in their muscles which do not lead to significant symptoms. These subclinical "patients" are not aware that they have a very mild Becker muscular dystrophy. The known patients seem to be just the top of the iceberg, and the accepted incidence of Becker boys at birth of 1:18,500 might be much too low.

As every laboratory has developed its own techniques, it is not easy to compare experimental results, therefore, standardization of the methods would be desirable. But this would be quite labor intensive and difficult to agree on. --- Also, one should be able to exchange cell lines and the oligonucleotides, but to synthesize them is very expensive. --- If one laboratory has a new system to check dystrophin or skipping, it should make it available to others, as development is expensive and time consuming. --- Regulatory agencies might insist on standardized methods.

The main goal of this meeting was to create an opportunity to exchange information, to stimulate cooperation, and thus to accelerate research for a therapy. It is planned to repeat this round table conference, perhaps yearly. And it was suggested to set up an international consortium to exchange information.

And finally: The large pharmaceutical company *Glaxo* is interested in performing clinical trials for Duchenne dystrophy. They have extensive experience in dealing with regulatory issues and may even help to develop the potential drugs.

On stop codon read-through: It is difficult to predict the response of individual patients to a gentamicin therapy. The response should be checked with the artificial system containing the exact mutation sequence of that patient. The researchers in Salt Lake City and in Orsay near Paris offer to do this for patients entering clinical trials. --- The response of a patient to gentamicin could also be checked in muscle cell cultures that can easily be made from fibroblasts, connective tissue cells from skin, of the patient.

Before a clinical trial, the gentamicin preparations have to be checked to make certain that they are active. This depends on the composition of their different components. --- The new dystrophin should be analyzed with antibodies in muscle biopsy material after treatment. It cannot be distinguished from normal dystrophin by routine analyses, because it is the same size. Just the one amino acid at the place of the stop codon is different. --- In some animal experiments, exon skipping has happened; then, the new dystrophin is shorter. --- Another clinical trial should be performed over a longer period of time.

Premature stop codons which appear after a deletion

causing a frame shift will also be affected by gentamicin, but that would not correct the reading frame and thus not lead to functional dystrophin. --- Whether the normal stop codons will always be respected as before is not clear. --- The toxic effect of gentamicin may partly be due to occasional suppression of normal stop codons and the incorpo-

ration of an incorrect amino acid into other proteins. --- The PTC124 compound seems to act by a mechanism that is different from the one used by gentamicin, and it does not seem to induce read-through of normal stop codons. Therefore, it is much less toxic.

The interview:

How long do we have to wait until exon skipping slows Duchenne down to Becker dystrophy?

In this interview, conducted on 17 January, Professor *Gert-Jan van Ommen* and Dr. *Judith van Deutekom*, of the University of Leiden in The Netherlands, and Dr. *Gerard Platenburg* of ProSensa, the company which will clinically develop the drug, answered questions, that were asked by *Günter Scheuerbrandt*, the author of this report. To better understand the interview, the report should be read first. The abbreviation “oligo” or “AON” means *antisense oligonucleotide*.

*From this interview, the families with Duchenne patients expect to get an answer, if that is possible, to their most important question: **How long do we have to wait until an effective therapy will be available for our boys?***

To give a short answer to this question is impossible, because to reach our goal, an effective and safe therapy, we have to take *one step after the other*, and we do not know how much time we will need for each step. We must now start working with our humanized mice. These mice have the full-size human dystrophin gene in their muscles, and as the next step we are trying to take out specific exons, like exons 45 or 52, so that we can use these mice to demonstrate that exon skipping works on the human gene in a living organism. That has not been done yet.

And the following, the second step, will then be real clinical trials with children. How long will it take until you can start them?

In the first clinical trial, we have to show that the mechanism works using an injection into a muscle. This will not yet be the method of the final therapy. That will probably be a systemic application into the blood stream. We are working very hard now to develop a clinical protocol, but also we try to manufacture the AONs themselves in a quality we would need to perform this type of work.

Will you have to modify these AONs so that you get them into the muscles systemically?

This is in full development and that will actually take a few more years. What should be clear, is that we need to demonstrate that exon skipping, which works in the mouse and in cell culture, works also in the muscles of children. But, we also have to show that we will not get side effects, and for that we will have to demonstrate that the degradation products we would expect are not toxic to a living organism. That part of the studies has to be done in animals. And if you want to know what the reasonable time would be for doing the experiments of injecting the AON for one of the best skippable exons into the muscles of only three or four patients in order to demonstrate that as a “proof of principle” in living patients, we think: *two or three years*.

It might go faster than that, but we may have unexpected problems with the regulatory authorities, or we may

have other problems for some reason that we never know beforehand. We know that by usually being optimistic by saying “in a year or a year and a half we will have the answer”, it never works like that. And so it is much more realistic to say, we will need two to three years for doing that study in a way that it really gives us a good and reliable answer, and three or four years to *complete* these first clinical tests with children with muscle injection to demonstrate that everything goes as we hope it will go.

Then, the time to start the next step will be much more uncertain, because we have to rely on developments in the field that we do not control ourselves. This step then is to get the antisense oligos into the muscle in an acceptable way, and everybody agrees that it will be systemically, through the bloodstream. Then we are talking about very different time frames.

About when will this third step, the first systemic trial with children, be completed?

To be honest, it will take at least six years from the start of the trial to complete it, if everything goes well. And people should realize that nowadays nobody really has a good proven protocol to actually perform that step. So there is an uncertainty even at the beginning of this clinical step, and the details are only now being addressed during this meeting and in many other therapeutic studies for other diseases where oligonucleotides are used.

But, if everything goes very well, can some steps which are prescribed by regulatory agencies be abbreviated?

We are developing a new type of drug whose side effects we do not know when we add it to a living organism. It gets everywhere in the body. And in every cell there are lots and lots of things that are precisely regulated. Many interact through specific short RNA molecules, the splicing mechanism for instance, or changing gene activities up or down or whatever. So there are many sorts of very short RNAs similar to our AONs whose effects we only now are beginning to understand. And what you ask from us is to make an estimate how long it takes when everything goes wonderfully well and everybody cooperates. And what will happen if we state a definite time, a certain day: everybody will see only that day.

It is very important that you say that so very clearly

because parents are looking desperately for that day.

Certainly, that is similar to the situation in diagnostic work when we say to parents that maybe they will have an answer in a week, but it also may take six months, then they start phoning after a week. And indeed, if we say that they cannot expect an answer before two months, and we call them up after only one month with a result instead of after two, they even become scared because they wonder what has gone wrong because it was going so fast. So it is much better not to raise hopes in an undue fashion.

Then you cannot give a more precise answer to the question about the waiting time?

Bill Gates has said: "People always overestimate what you can achieve in two years, and underestimate what you can achieve in ten years". There is a middle way, and we would be disappointed if the whole procedure took longer than 10 years until the first truly encouraging results with systemic administration are obtained.

However, people should realize that 10 to 15 years is the average development time for a normal medication, while here we are dealing with many unknowns. Some things might go much better, and some things might go much worse. But we think, 10 years would be a reasonable estimate for seeing the first promising application of the antisense work for Duchenne. Of course, we hope it will take less, and there is a chance that it will take less.

So one can say that for those children, who are now born with Duchenne dystrophy, their chance for a therapy is higher than for those who are now 10 years old. But will exon skipping be applicable later for patients who are 20 or more years old?

It is possible that we could slow the disease down even then, but we would be amazed if we would really have a complete cure for 15 – 20 year old patients, because a lot of their muscles would already have been replaced by fat and connective tissue. But the situation will depend on the course of the disease of each individual patient, and that may be quite different. Some patients become ill very rapidly and then become weaker slowly, and some patients deteriorate more steadily. We just don't always understand yet why things go wrong and how they go wrong.

But everybody just hopes for a complete revival of everything. However, once Duchenne patients are older than 15 to 20 years, they have gone through many stages of their life. And some would understand that they will never get much better than they are, but also that they would not get much worse if exon skipping works for them. So, for the older Duchenne patients, after another ten years we will probably be able to make the process go much slower or even to bring it to a standstill, but quite probably not to get them, from one day to the next, out of their wheelchair and see them walk away.

Can these antisense oligonucleotides be made automatically and in sufficiently large quantities? And will you have to make an individual drug for each patient whose structure will depend on his particular mutation? So you will have to "custom make" many different AONs?

We will probably not need to make them "custom made" for each individual patient, some of them will be used for an entire group of patients. We can make them automatically in a very pure quality, so they will be safe.

And will they be quite expensive when they come into

the pharmacies?

It is very hard to make a comment on that. The clinical grade AONs for the experiments in patients are extremely expensive. That is why clinical trials cost so much.

Medication for another genetic disease, Gaucher's disease, costs 200.000 dollars a year. And there are very few patients in the United States who do not, in one way or the other, find the money to get the treatment. It is either through the patient organizations or it is from rich parents or health insurance or whatever. And not many people are going to be left behind. So, it is difficult to say whether a treatment is expensive or cheap, because that depends on what the patient is getting. If the price is 50,000 euros per year for an extra life span of 20 years with an improved quality of life, then insurance or parents or whoever will probably find the money.

The last question: Parents are desperate, they are trying everything, they are trying creatine, which is not really working, then they try coenzyme Q10 or Abyssinian tea, or they even go to those miracle doctors in Kiew, who ask for 60.000 euros just for the first visit. What would you as scientists say to that?

That is something that people really have to decide for themselves. We can understand that they are desperate, we can understand that they would want to go in any direction that may be hopeful, but we also think that scientifically there cannot be a basis for a treatment as proposed in Kiew to work. We do not have Duchenne children ourselves, but if we had one, we would keep our money for other things.

We have time for some final words.

To finish this interview, let us stress that we really need to take *one step at a time*. And that is why we first worked on the mouse, then we did the in-vitro work with human muscle cells, then we introduced human dystrophin genes into the mouse, and we are now making human mutations there, and finally, after all these preparations, not earlier, will we work with Duchenne boys, first with local injections, then with a systemic approach, which, we hope, will be the final therapeutic procedure.

But a boy is not a big mouse, and a mouse is not a human weighing 30 grams. They are very different. If a boy has no dystrophin, he has Duchenne. If a mouse has no dystrophin, it is very mildly affected. And so one has to understand that there are differences between mice and children. That is why what we have done in the mouse needs to be repeated in humans. And it is important that we do the first clinical step to show that by injecting the AONs into the muscles we really get what we expect, even if that is not going to be the final direction of the therapy. And that is in itself reason for further hope, that there will be always another next step.

We, as scientists who get the trials going, need those steps to show that we are making progress, but one step at a time. Sometimes it is very difficult to actually look over the horizon, but if we take only one step at a time there will be a moment when we can actually see our goal on the horizon and coming closer and closer.

It was important for you to say that. All Duchenne families are thankful for your and your colleagues' efforts to bring exon skipping to the clinic and to their children hopefully faster than in ten years.

An attempt to come to a conclusion:

I, the writer of this report, *Günter Scheuerbrandt*, am not a medical doctor – I am a biochemist –, and I am not actively engaged in research for a therapy of Duchenne dystrophy. But I know the situation, the hope, and the despair of the families with Duchenne boys quite well. My 30-year long experience with our early detection program for this disease in Germany has brought me into contact with many families, so I know what is going on. This may allow me to come to a conclusion after the results of this remarkable meeting on the two therapeutic approaches, *exon skipping* and *stop read-through*.

Other research approaches for a therapy like transfer of stem cells, transfer of the entire dystrophin coding sequence, its cDNA, or shorter versions of it with adeno or adeno-associated viruses, upregulation of utrophin and many others are also actively pursued – one, in France, is even being clinically tested – but they are probably not as close to a functional and useful therapy as exon skipping seems to be today

However, exon skipping will not be a final therapy which will cure the disease for good and make the boys walk again. It will only slow down its relentless progression and thus give the boys more time to wait for a complete cure. But about 10% of the boys will be left behind when exon skipping improves the life of the majority, of 90% of them. And codon read-through will help only about 10%, those with the appropriate mutation in their dystrophin gene. Once it works, it will probably not be 100% effective, the amount of new dystrophin will be lower than normal and that would also mean a kind of Becker dystrophy.

And for both methods, a single application will not be sufficient. Repeated applications, perhaps for the rest of the boys' lives, will be necessary to maintain the therapeutic effects. That is not what one expects of a real drug, a drug that cures a "normal" disease like tuberculosis or stomach ulcers. However, Duchenne muscular dystrophy is not a normal disease. Therefore, a partial cure would already be an extraordinary medical victory.

In the interview, the Dutch researchers say that it may take 10 years, more or less, until a personalized exon skipping drug can be ordered at the pharmacy for a boy with Duchenne dystrophy. That will give hope to those families whose sick son is not yet 5 years old. But for the families with 10 or 12 year old boys, that will mean their disease will not be slowed down before they are about 20, when most of their muscles have disappeared. And it is not likely that another method will be ready in less than 10 years. This news from this meeting is disappointing, which might not quite be so serious when it turns out that the waiting time will be perhaps less. After all, predictions of this kind can never be precise.

So, will the great leap forward in Kobe in Japan be the answer? Or the trial in Naples? Perhaps, but only perhaps, because one single patient was treated in both places. So it may have been a lucky unique result which cannot be generalized. And if something goes wrong, if the treated boys become more ill than they were before the treatment? Or if something more drastic happens? Then the entire research field, all the efforts to manipulate a gene in this

way, could be affected, with consequences as serious as a general stop of all clinical trials by the regulating authorities everywhere. On the other hand – the Japanese boy might really have his life threatening disease permanently transformed into a much more benign one! Or the Italian boy remains without symptoms. After all, the Japanese and the Italian researchers are experienced scientists who know what they are doing. In a year or so, we will know whether these experiments were successful, and that could create a completely new situation.

So, that is my opinion – and I may be wrong. But I am not wrong in saying that the scientists who presented their work at the meeting and all their co-workers deserve our sincere thanks for their efforts and their dedication in spite of all the difficulties they encounter on their way to the therapy that we are so desperately waiting for.

And what do the parents say? *Nick Catlin* from the British Duchenne Parent Project (PPUK) and father of a still young Duchenne child, expressed his feelings and certainly those of many other parents at the end of the meeting:

"We already give our kids chemicals like steroids that we know are toxic and can have harmful side effects. We as parents are doing this because there is absolutely nothing else. I certainly do not want to give my boy anything poisonous. However, we can see from the presentations here at Monaco that the oligonucleotides do not appear to be toxic in animals, and the early results of the Japanese study tell us the same. What parents want to see is this research moving forward quickly with clinical trials. This would offer families real hope for the future. We should not be hung up at this stage about toxicity. Time is not on our side and we want something that is an improvement on drugs like steroids.

We parents understand the difficulties that the researchers have to overcome, but we really do need to see clinical trials of the antisense therapy happening as soon as possible. The funding offered by *Elizabeth Vroom* to the Dutch group through the United Parent Projects will be absolutely fantastic. It would be an absolute tragedy not only for my son's generation but for the generation of young boys being born every day around the world if this research is held back for six months or another year or two, because the money is not available. You can be sure that we won't give up trying to provide the money!

Seeing what is happening to my son every day, losing his muscle strength and having difficulties keeping up with other kids, this research gives me realistic hope. It has been exciting for me to come to the conference and I will be going back to London with a lot more hope. And it is great that parents from all over the world are determined to fund this research work. That is great credit to the parents' representatives who are here: *Pat Furlong* from the US, *Elizabeth Vroom* from Holland, *Luc Pettavino* from Monaco, *Christine Dattola* from France, *Sally Hofmeister* from Germany, *Fillipo Buccella* from Italy, *Jenny Versnel* from the UK, and *Helen Posselt* from Australia and all the other parents that raise money and keep up the fight for a cure for Duchenne dystrophy. Let's please keep going forward."

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Participants of the round table conference

Scientists:

The scientists are listed with their abbreviated addresses and without any titles. Most of them are professors and all have an MD or PhD. Those who presented their work have an * in front of their name. The others were chair persons of the sessions and/or participated in the discussions.

- *Irene **Bozzoni**, University La Sapienza, Rome, Italy
- Kate **Bushby**, University of Newcastle, UK
- Oliver **Danos**, Genethon-CNRS, Evry France
- *George **Dickson**, Royal Holloway University, London, UK
- *Judith C. T. **van Deutekom**, Leiden University Medical Center, Leiden, Netherlands
- *Kenneth **Fischbeck**, National Institutes of Health, Bethesda MD, USA
- *Kevin M. **Flanigan**, University of Utah, Salt Lake City UT, USA
- *Luis **Garcia**, Genethon-CNRS, Evry, France
- *Jean-Claude **Kaplan**, Hôpital Cochin, Paris, France
- Hanns **Lochmüller**, University Munich, Germany
- Qui Long **Lu**, Hammersmith Hospital, London, UK
- *Masafumi **Matsuo**, Kobe University, School of Medicine, Kobe, Japan
- Richard **Mulligan**, Harvard Medical School, Boston MA, USA
- *Gertjan B. **van Ommen**, Leiden University Medical Center, Leiden, Netherlands
- *Terrence **Partridge**, Hammersmith Hospital, London, UK
- Gerard **Platenburg**, ProSensa Company, Leiden, Netherlands
- *Luisa **Politano**, 2nd University of Naples, Naples, Italy
- *Thomas **Rando**, Stanford University School of Medicine, Stanford CA, USA
- *Jean-Pierre **Rousset**, University of Paris XI, Orsay, France
- *Daniel **Schümperli**, University of Berne, Berne, Switzerland
- *Lee **Sweeney**, University of Pennsylvania, Philadelphia PA, USA
- *Steve **Wilton**, University of Western Australia, Perth, Australia

Parents' representatives:

Fillipo **Buccella**, Duchenne Parent Project, Italy
Nick **Catlin**, Duchenne Parent Project, UK
Christine **Dattola**, Duchenne Parent Project, France
Patricia **Furlong**, Duchenne Parent Project, USA
Sally **Hofmeister**, Aktion Benny & Co, Germany
Rod **Howell**, Muscular Dystrophy Association, USA
Peter **McPartland**, Duchenne Parent Project, UK
Luc **Pettavino**, Duchenne Parent Project, Monaco
Helen **Posselt**, Duchenne Parent Project, Australia
Jacques **Salama**, Association Française contre les Myopathies, France
Elizabeth **Vroom**, Duchenne Parent Project, Netherlands

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A report on all research approaches with results up to August 2003 can be seen in English, German, French, and Spanish at <http://www.duchenne-research.com>. Those who wish to receive future updates of the reports should send their e-mail address to Günter Scheuerbrandt.

Exon Skipping, an Example

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

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

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

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

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

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

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

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

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