Research approaches for a Therapy of Duchenne Muscular Dystrophy.

Part 1: Exon Skipping.

This is the first update of my report on exon skipping, originally published on the 30th of April 2009. Exon skipping is the most advanced genetic technique for an effective therapy of Duchenne muscular dystrophy. It is one of my many reports which I, Günter Scheuerbrandt, a biochemist in Germany, am writing for you, the Duchenne boys and young men, their families and care givers, who wish to know how the research work of many scientists and clinicians is progressing. All my reports and this one too, are not scientific publications with many difficult words, because I have tried to write them in a way that will let you understand what is happening for you in the laboratories even if you have not studied modern biochemistry and genetics.

This text on exon skipping is the first part of the entire report about all research approaches for a therapy of Duchenne muscular dystrophy. The remaining parts will be published during the next months. They will each contain the latest news on several other therapeutic approaches like the transfer of the dystrophin gene into muscle cells, the use of stem cells, the upregulation of utrophin, and on a number of other pharmacological methods.

Every one of the new reports will be individually updated from time to time with newly published results and the news presented at scientific meetings. As I am a biochemist and not a medical doctor, my reports contain only information on therapeutic research but not on the medical treatment and management of Duchenne patients. Compared with the first version, this updated text contains summaries on the results of the local British and the systemic Dutch clinical trials for skipping exon 51, the very positive results of using peptide-conjugated antisense-oligos, the genetic exon skipping with the U1 splicing factor, the octa-guanidine-type of antisense oligos, and some corrections and additions throughout the text.

I have written this text as all my other reports first in English and will translate it into German a few weeks later. Ricardo Rojas in Mexico will make the Spanish translation. You can see all my recent reports and interviews in these three languages on my internet pages www.duchenne-information.eu. Translations into some other languages, Japanese among others, are also available.

In the summaries, I only mention the names of the heads of laboratories, although they have colleagues and students working as a team on the projects reported here, but it is impossible to list them all. I have written the names of the scientists without their academic titles, but most of them are professors and all have an MD or PhD degree.

References to some of the most important publications are given at the end of this report. They are indicated by numbers in parentheses, e.g. (12), at the places in the text where they belong.

If you have questions concerning exon skipping and other Duchenne research, please send me an e-mail to gscheuerbrandt@t-online.de in English, French, German, or Spanish, I will try to answer all of them, but only in English or German.

How genes make proteins

**Genes** are functional units of the genetic material **deoxyribonucleic acid, DNA**. Its structure looks like an intertwined ladder, the **double helix**, which was described in 1953 by James Watson and Frances Crick (see next page). Each rung of this ladder contains two of four different small molecules, the **bases**: adenine, guanine, thymine, and cytosine (A, G, T, C).

We can call them the **genetic letters**. The rungs can only contain two base combinations, the **base pairs** A-T and G-C. If, for instance, GGCTTAATCGT is the sequence of these bases on one strand of the DNA, the sequence on the opposite strand must be **complementary** to it. A is opposite T and G opposite C because then they just fit between the strands:

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-GGCTTAATCGT-
  ||||| |||||
-CGAATTAGCA-
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This sequence of the bases, of the **genetic letters**, is the **genetic information** for the development and maintenance of a living organism, and it is passed on from one generation to the next.
Most of the genes carry the instructions for the biosynthesis of proteins. In the cell nucleus, the genetic instruction of active genes is expressed, it is copied, transcribed, to another genetic substance, the premature messenger ribonucleic acid or pre-mRNA, also called the transcript. Most genes consist of active or coding regions, the exons, which contain the information for the proteins, and often much longer introns, which do not contain only “genetic junk”, as one once thought, but also important information for the control of gene activities. The ribonucleic acids, RNAs, use the base U, uracil, instead of the similar base T of the DNA.

After transcription and still inside the cell nucleus, the introns are removed from the pre-mRNA, and the exons spliced together to form the messenger RNA, mRNA, which then contains only the coding regions, the genetic information for the synthesis of a protein. This mRNA then leaves the nucleus and moves to the cytoplasm outside the nucleus. Splice sites are specific sequences inside the exons and at the borders of exons to introns which are essential for the correct removal of the non-coding intron sequences from the pre-mRNA. The splicing itself is accomplished by spliceosomes, a complex of many proteins and small RNAs.

To show you how far scientific research has advanced to understand the function of living cells, I am enclosing here stereo pictures of one of the 5 structures which form the spliceosomes in human tissue. The molecular structure of this complex, called U1 snRNP, small nuclear ribonucleoprotein, was published in March 2009 in the journal Nature (1). This U1 complex alone consists of 10 proteins and one RNA. You can see the 3-dimensional structure by focussing your left eye on the left picture and the right eye on the right picture without any instrument. You will then see 3 pictures, the one in the middle quite spectacularly in three dimensions. The orange line U1C-70 K is the most important of the proteins which recognizes the exon-intron borders and orchestrates the splicing reaction.

These illustrations are highly abstract. In reality, there are no colors and everything looks like a grey jelly. I mention these very new research results not only to give you an example of how modern science is presented, but also because a similar complex, a modified U7 snRNA is already been used for exon skipping with gene transfer (see page 10).

The genetic code. For the translation of the language of the gene into that for the proteins, the genetic information of the mRNA is written in genetic words each consisting of three consecutive bases, the codons, which specify, with three exceptions, one of 20 different amino acids, the building blocks of the proteins, according to the genetic code. There are 64 different code words of 3 bases each. Here are a few examples:

GUU = valine, AGC = serine, AUG = methionine, CCA = proline, UUU = phenylalanine, GCA = alanine, GCG = alanine, etc.

Most amino acids have more than one RNA code word. There are no spaces between the codons. Therefore, the first code word of the coding sequence for a protein – it is always AUG – defines a reading frame. If this frame shifts by one or two letters, the code words change their meaning, they then specify different amino acids. This is very important for understanding how exon skipping works.

In the ribosomes, the genetic code words of the messenger RNA are read and translated into the language of the proteins, which are built of many, often thousands, of amino acids. The three exceptions mentioned above are the words UAA, UAG, and UGA, which are stop codons, where the assembly of the protein in the ribosomes comes to a halt.

You can see a film showing a gene making RNA and the ribosome assembling a protein by using the information in the RNA as fast as it happens in nature at: www.youtube.com/watch?v=D3IOXt4MrOM&feature=related – click on this address + Strg key and then click on the button at the right of the HQ button to see the film full screen. If you have loudspeakers, turn them on.

The speed of the ribosomes reading the genetic information of the mRNA and assembling the amino acids to the chain of a protein has recently been measured (26). On the average, it takes 2.9 seconds to add one more amino acid to the growing chain. As the dystrophin protein consists of 3,685 amino acids, it takes about 3 hours to assemble the entire chain of this very long protein.
Dystrophin gene and protein.

**Duchenne muscular dystrophy** affects only boys – about each 3,500th newborn – because women, when they are genetic carriers, transmit it, on the average, to half of their sons. Sometimes, it appears spontaneously in a new family. This still incurable hereditary disease is being caused by mutations of the by far largest of our 20,488 genes, the dystrophin gene.

The next illustration shows the location of the gene on the short arm of the X chromosome. Its DNA consists of 2,220,223 genetic letters, which are grouped in 79 exons, the active sections. Indicated are also the 7 promoters, the starting regions for the production of the full-length and the 6 shorter versions of the protein. After splicing, the mRNA contains only 11,058 genetic letters, only 0.5% of those of the entire gene. In the ribosomes, the protein **dystrophin** is assembled according to the genetic information in the mRNA from 3,685 amino acids which are being brought to the site of synthesis by another kind of RNA, the transfer or tRNAs.

The dystrophin protein has a rod like shape with 24 repeated amino acid sequences separated by 4 hinge regions. Its two end regions are called the N and C terminals, and there is also a region with many cysteins, sulfur-containing amino acids. In the right corner is a cross section of muscle tissue with the dystrophins in the cell membranes made visible with fluorescent antibodies.

**The size of the dystrophin gene and protein.** The double-helix structure of the dystrophin gene is 0.75 mm long. Together with the other about 20,000 human genes, it fits into a cell nucleus of about 0.01 mm diameter because the genetic material is extremely tightly packed. One molecule of the full-length dystrophin protein is much shorter than its gene, it is 125 nm, nanometers, = 0.000125 mm, long, 8,000 of them laid end to end in a straight line would cover just one millimeter. And in one gram of muscle, there are 114 billion dystrophin molecules.

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

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

**Duchenne boys have either none or very little dystrophin in their muscle fibers.** When its protective and organizing effects are missing, the muscle contraction causes the rupture of the muscle membranes, and this allows relatively large amounts of calcium to flow into the fibers. The excessive calcium activates enzymes like calpain and other proteases that break down muscle proteins and initiate cell death programs, apoptosis. The consequences are a chain of events like inflammation and activation of fibroblasts which lead to fibrosis, scar tissue that slows down muscle regeneration and causes the typical symptoms of older Duchenne patients.

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

But not only the skeletal muscles suffer when dystrophin is missing, but also the smooth and heart muscles. Damage to the heart muscles produces cardiomyopathy,
and the weakness of the smooth muscles has many con-
sequences, among them the reduced ability of blood
vessels to relax, when blood flow increases, leading to
respiratory and other problems, and also the gastro-
intestinal tract is affected when the motility of the intest-
tines is reduced. So the damage of just one gene can af-
flect large parts of the body.

Exon Skipping

The task of research. A healthy 5-year old boy weighing
30 kg has about 12 kg muscles which contain 1.5 quadri-
lion (1.5 x 10^{15}) dystrophin molecules. A 5-year old Du-
chenne boy has only 6 kg muscles left which practically do
not contain any dystrophin, because the information of the
damaged gene cannot be correctly read during the biosyn-
thesis of the protein. In order to make his remaining mus-
cles function again, at least to a certain extent, about 30%
of the normal amount of dystrophin must reappear and be
present during his entire life (2), this would be 200 trillion
(200 x 10^{15}) molecules in his 6 kg of muscles. The new
dystrophins don’t have to have exactly the same length and
form of the normal one in the muscle, they can be shorter,
but they must be able to work properly.

Exon skipping, a genetic Duchenne therapy. At a dis-
cussion in the mid-90s, Gertjan van Ommen of the Uni-
versity of Leiden in the Netherlands explained to me how a
genetic therapy could accomplish this task for a long time
without serious side effects. It is now called exon skipping
and has been developed during the last 15 years by his and
other research groups, above all in Japan, Australia, the
UK and the United States to such an extent that this proce-
dure is not only being tested on sick mice and dogs but al-
ready on Duchenne patients.

Local and systemic clinical trials – on one single mus-
cle or on all of them – for skipping exon 51 of the pre-
mRNA in Duchenne boys have been and are being per-
formed with the help and partial funding by the Dutch
company Proensa in Leuven, and Gothenburg and by the
American company AVI BioPharma in London and
Newcastle under the direction of the MDEX Consortium.
In addition to van Ommen, the teams around Judith van
Deutekom and Annemieke Aartsma-Rus in the Nether-
lands and around Kate Bushby and Francesco Muntoni in
the UK should be mentioned here, too.

Exon skipping means “jumping across exons”. Exons are
the active sections of a gene. In the dystrophin gene of
most Duchenne boys, one or more exons are missing, or
they are duplicated or have mistakes in the sequence of
their letters. This interrupts the reading process of the ge-
netic information for the protein biosynthesis so that no
dystrophin can be made. These mistakes can be corrected,
that is, the protein synthesis can be restored again if one
blocks one or more of the still present neighboring exons
in such a way that the mechanism that joins exons skips
over them and thus does not use them anymore (3).

For this blockade, antisense oligonucleotides, AOs, are
needed. They are short pieces of genetic material, about 20
to 30 genetic letters long, with a special sequence, so that
they can attach themselves to the complementary exactly
fitting sequence of the exon to be skipped. AOs have now
been made, mainly by Steve Wilton at Perth in Australia
for all 79 exons (4) and by Annemieke Aartsma-Rus in
Leiden in the Netherlands, for 39 exons, each with another
structure which blocks only the one targeted exon but not a
single other one in the many thousand other human genes.

Duchenne is converted into Becker dystrophy. Because of
the missing exons – those deleted by the mutation and
those skipped in addition –, the amino acid building blocks
determined by these missing exons will also be missing in
the newly made dystrophin. Thus, the new dystrophin will
be shorter than normal but it will often still be able to pro-
tect to a certain extent the muscle cell membranes. There-
fore, the symptoms of the disease will be milder, the mus-
cle degeneration will proceed more slowly, and the life ex-
pectancy should increase significantly up to normal in
some cases. The Duchenne dystrophy would then have
been changed to the mild variant of this disease, to Becker
muscular dystrophy.

A therapy but no cure yet. As the patients will still be
handicapped after the treatment, however significantly less
than before, exon skipping cannot be a cure of the disease
but only an effective therapy. With this method of gene
technology, the damaged gene itself will neither be replac-
ited nor repaired, but only the mechanism of reading its in-
formation will be corrected.

Animal experiments. Exon skipping has been tested with
success on dystrophic mice and dogs, locally by injection
of the AOs into a single muscle, or systemically into the
blood circulation so that all muscles are reached, also those
of the heart and the lung function. In most of the systemic
experiments, the muscle functions could be significantly
improved. One of the most important of these pre-clinical
experiments with systemic injections of the AOs into the
blood circulation of dystrophic mdx mice has been pub-
lished by Terence Partridge and colleagues in 2005 (5).

The mutations of the dystrophin gene. There are many
reports in the scientific literature about the percentages of
the different types of the dystrophin mutations among all
Duchenne patients. The publication by Annemieke Aarts-
ma-Rus and colleagues in 2006 (6) is based on more than
4,700 mutations listed in the Leiden Duchenne muscular
dystrophy database and thus seems to be the most reliable.
According to this publication, deletions of one or more
exons make up 72% of all mutations. Duplications of one
or more exons are found in 7% of all patients. 20% have
point mutations, that is, very small deletions or insertions
of one or a few genetic letters, and in the remaining 1%,
several rare mutations are found like those disrupting
splice sites or rearranging large parts of the gene structure.

The authors conclude that for 91% of the patients the
reading-frame rule holds true, meaning that out-of-frame
mutations cause Duchenne and in-frame mutations Becker muscular dystrophy. They say also that in most of the patients, whose mutations seem to be exceptions to the reading-frame rule, the structure of their mRNA may indeed follow this rule. However, in most cases, the mRNA sequence is not determined when a genetic analysis is performed. Before an exon skipping treatment is started, it may be advisable to confirm in a tissue-culture experiment that this treatment really produces an in-frame mRNA.

**Applicability of exon skipping.** Although all Duchenne patients have more or less similar clinical symptoms, there are many different causes of their disease, because the mutations of the very large dystrophin gene can happen on many different sites. Therefore, exon skipping is **mutation specific.** It will be a personalized therapy. Each patient will need a specialized AO, but each AO can often be used for a group of patients with different mutations that need the skipping of one or more particular exons.

**Annemieke Aartsma-Rus** and her colleagues have reported the applicability for skipping one or two exons in Duchenne patients with deletions, point mutations and duplications reported in the Leiden Duchenne Muscular Dystrophy pages as of the 11th of March 2008. They analyzed the genetic and clinical data of 4,770 patients and listed 120 groups of patients needing the skipping of one or two particular exons according to their percentage relative to Duchenne patients with all kinds of different mutations. Here is a list of the percentages of the 8 largest groups:

<table>
<thead>
<tr>
<th>Rank</th>
<th>Exon to be skipped</th>
<th>% of all patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51</td>
<td>13.0</td>
</tr>
<tr>
<td>2</td>
<td>45</td>
<td>8.1</td>
</tr>
<tr>
<td>3</td>
<td>53</td>
<td>7.7</td>
</tr>
<tr>
<td>4</td>
<td>44</td>
<td>6.2</td>
</tr>
<tr>
<td>5</td>
<td>46</td>
<td>4.3</td>
</tr>
<tr>
<td>6</td>
<td>52</td>
<td>4.1</td>
</tr>
<tr>
<td>7</td>
<td>50</td>
<td>4.0</td>
</tr>
<tr>
<td>8</td>
<td>43</td>
<td>3.8</td>
</tr>
<tr>
<td>1–8</td>
<td></td>
<td>51.2%</td>
</tr>
</tbody>
</table>

The entire list has been published in March 2009 (7). If you would like to have this publication, please write to me.

As shown in this short list, 13.0% of all patients will need the skipping of their exon 51, the anti-51 AO thus is the potential skipping drug for the largest group of all Duchenne patients. For this reason, the Dutch and British scientists try to skip just this particular exon in their first clinical trials to help this group of patients as soon as possible. And the Dutch company Prosensa is developing the skipping of the exons in the groups 2–8, provided the exon-51 trials are successful. With the AOs against the exons in this priority list, more than half of all Duchenne patients could be treated.

**Double and multi-exon skipping.** Many of the Duchenne dystrophies caused by deletions, duplications, and point mutations will need the skipping of two or more exons to restore the reading frame.

By theoretical considerations it was even predicted that a simultaneous skipping of the 11 exons 45 to 55 would produce a Becker dystrophy with very mild symptoms in up to 63% of all Duchenne boys (8).

**Annemieke Aartsma-Rus** and her team have tried to remove in cell culture these 11 exons from myoblasts obtained from a healthy person and from two Duchenne patients with deletions of exons 48-50 and 46-50. Cocktails of 2′O-methyl AOs against the human sequences of each of the 11 exons were used in different combinations including a mixture of all 11 AOs. Different intermediate partially skipped mRNAs were obtained at low levels and occasionally also the mRNA with all targeted exons deleted. Irregular splicing processes of the rather short exons bordered by very long introns may have been the reason for the difficulties to skip the 11 targeted exons in a consecutive way.

The authors conclude that this approach is theoretically promising for producing a very mild Becker dystrophy but that the current state of this technique does not sufficiently support the clinical development of multi-exon 45-55 skipping (9).

Because dystrophic dogs need the simultaneous skipping of two exons, experiments with them would open the way to the development of multi-exon skipping for Duchenne boys. The results of the first successful skipping of 3 exons in dogs have been published in March 2009 (10) and are summarized here. They created much interest and attention from parents and patients. However, they only mean that skipping 2 or 3 exons or a few more is and will be feasible, but skipping the 11 exons 45-55 will not be possible for the time being as explained before.

**Multi-exon skipping in dystrophic dogs. Eric Hoffman and Terence Partridge of the Children’s National Medical Center in Washington, Shin’ichi Takeda of the General Animal Research Facility in Tokyo, and their colleagues developed a cocktail of morpholino antisense oligos (AOs) for multi-exon skipping in dystrophic CXMD beagle dogs (10). In contrast to the mdx mice with their mild dystrophic symptoms, these dogs are physically handicapped and they are much larger than mice. Thus experiments with them give results that would likely be similar to those obtained in clinical studies with Duchenne boys. And experiments lasting several years can be performed with dogs, because they live much longer than mice.

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

Before the experiments with live dogs could be started, preliminary tests in tissue cultures with isolated myoblasts from these dogs were necessary. Four AOs with sequence of 24 and 25 bases were constructed against sequences inside exons 6 and 8 and against the borders of exon 6 to intron 6 and of exon 8 to intron 8. They had to be 2′O-methyl AOs which are electrically charged and enter cells in tissue culture tests more efficiently than electrically neutral morpholinos. These AOs were used alone or as a cocktail of a mixture of all four.

The results of the cocktail experiments showed, that
four days after the myoblasts had produced myotubes — another stage of muscle development —, the mRNA sequence joined the end of exon 5 directly to the beginning of exon 10. That means that in addition to the deleted exon 7 and the desired skipping of exons 6 and 8, exon 9 was also skipped although no AO was used against this exon. It is not yet understood why this happens. But skipping of exon 9 alone does not shift the reading frame, so this extra skipping does not affect the therapeutic outcome of this type of multi-exon skipping. The production of dystrophin in these isolated myotubes could also be confirmed. In the experiments with the single AOs, it was shown that the one against the border of exon 8 to intron 9 did not affect the outcome of the skipping, so in the experiments with living dogs, the cocktail contained only three AOs, designated Ex6A, Ex6B, and EX8A.

The next step was the injection of 0.5 and 1.2 mg of the three-AO cocktails, containing equal amounts of each AO, locally into one single tibialis anterior (shin) muscle of a 6-month old and a 5-year old living dog. This time, both types of AOs were used. 2'O-methyls and morpholinos. Two weeks later, tissue from around the injection sites was obtained by biopsies. Between 61 and 83% of the mRNA in the muscle fibers around the injection site had a sequence missing the exons 6, 7, 8, and 9. With the 1.2 mg dose of morpholinos, a practically normal level of new dystrophin protein was seen, and the results with the 2'O-methyl cocktail were similar. The structure of the dystrophin-positive cells was significantly improved, and the results with the young dog were better than with the older one.

Thus the quality of the muscle influences the amount of the dystrophin that can be produced, again an indication that exon skipping, once it becomes available, should be started as early as possible. It was also noted that, whereas in tissue culture a single AO against exon 6 could lead to efficient skipping of exons 6-9, this did not happen when the same single morpholino was injected into the dog’s muscles, where the complete cocktail was required. This means that one cannot necessarily rely on tissue cultures as a means of screening effectiveness of a given AO to induce skipping.

For a systemic treatment, performed in Shin’ichi Takeda’s Animal Research Facility in Tokyo, three 2-month old dogs were treated by injecting the three-morpholino AO cocktail into their leg veins. The first dog received doses of 120 mg/kg once a week for 5 weeks, the second the same dose every second week 11 times for 5.5 months and the third 200 mg/kg once a week for 7 weeks. Two weeks after the last injection, many of their muscles were examined.

In all the tested muscles of each treated dog, new dystrophin was found in up to 50% of the normal level, but some muscles, especially the heart muscle fibers, had only trace amounts of new dystrophin. The muscles of the dog which received the largest dose of the cocktail showed an average level of 26% of the normal amount of dystrophin, which, based on earlier findings, is sufficient for normal muscle function. The new dystrophin had the amino acids missing which are coded for by the m-RNA sequence of the exons 6, 7, 8, and 9. This proves that in addition to the missing exon 7 the two targeted exons 6 and 8 were skipped and also, for unknown reasons, exon 9.

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

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

You can see one of the dogs in two short films before and after the treatment on one of my internet pages: http://www.duchenne-information.eu/home-en.htm. If you do not understand what the people say, who are taking care of the dog, then you do not speak Japanese.

Exon skipping to repair duplications. Duplications of one or more exons causing a shift of the reading frame have happened in about 7% of all Duchenne patients. In principle, they can be repaired by exon skipping also, if it were possible to remove the one additional set of the duplicated exons without touching the first, the original set of exons. Such an exon-skipping treatment would then not only be a therapy but a real cure, because, after the removal of the extra exons, that mRNA would have its normal structure with all exons present only once, and the new dystrophin protein would have the normal size.

But the situation is not simple, mainly because it is not easy to direct an AO to only one of two identical exon sequences. Annemieke Aartsma-Rus and her colleagues have done laboratory experiments in cultured muscle cells from Duchenne patients with duplications (11).

They were able to correct an exon-45 duplication by skipping one of the two exons, and up to 80% of the muscle fibers contained normal dystrophin two days after this in-vitro treatment. On the other hand, it was impossible to remove only one of the two exons 44 in the isolated muscle fibers from two Duchenne brothers. However, in this case, skipping of the two exons 44 and in addition of exon 43 would restore the reading frame. The attempt to correct the larger duplication of exons 52-62 was not successful.

At the request of a family whose son had the exons 8-11 duplicated, Annemieke answered to explain the difficulties: “We would need a combination, a cocktail of AOs targeting all four exons 8-11. But these AOs cannot discriminate between the original and the duplicated exons. So the result will be skipping of either the original exon 8, the duplicated exon 8 or of both exons 8. Or it may skip the original exon 9, the duplicated exon 9 or both, or a combination of exons 8 and 9, and so on. Thus there are many possibilities only one of which — skipping the dupli-
cated exons 8, 9, 10 and 11, but not the original exons 8, 9, 10 and 11 – will restore the reading frame. The effect is diluted. Simply increasing the amount of the AOs in the cocktail will not change the situation. We are trying to find solutions for these problems but things are not as straightforward as with deletions. We do not know if, and if so, when exon skipping will be applicable for large duplications’.

**Exon skipping to repair point mutations.** Point mutations are small changes of one or several genetic letters in the gene itself. If the mutation has added or deleted one single letter, then the reading frame is shifted. Or one letter has been exchanged against another, then the reading frame is not shifted, but the code word now may mean another amino acid. If this exchange does not disrupt the structure of the dystrophin, then nothing happens. But if one of the three stop codons, TGA, TAG, or TAA, has appeared, then – although the reading frame is not shifted – one of the three stop codons, TGA, TAG, or TAA, has appeared, then – although the reading frame is not shifted – the protein synthesis is halted at such a premature stop sign, and the result is Duchenne dystrophy. It may be possible in the future that such a stop mutation can be overcome with the drug PTC124 which is already in clinical trials. Or it can be repaired by skipping the particular exon containing the stop codon if the exon has the correct borders so that its deletion would not shift the reading frame. Or, if this does cause a frame shift, a neighboring exon would have to be skipped in addition (12).

**Exon skipping to repair rare mutations.** Even shifted reading frames caused by rare mutations at the border regions between an exon and a neighboring intron, the so-called splice sites, could be restored by exon skipping. As an example, I am quoting here again Annemieke Aartsma-Rus explaining how an exchange of the base A to T at the beginning of intron 46 causes rather mild Duchenne symptoms in Tomas, a patient from Argentina:

“This mutation will probably lead to a partial or full spontaneous skipping of exon 46 in the mRNA which cannot be seen at the DNA level by a genetic test. This splice site is normally rather active for this exon, but due to this mutation, there is a very severe inactivation of this site. I can imagine that exon 46 is skipped in the mRNA of this patient. This, however, is a prediction and we cannot know for certain whether and how much exon 46 is skipped until muscle mRNA is analyzed. In case the exon-46 skipping is not complete, Tomas will have low levels of dystrophin, and this may be the reason for his mild form of Duchenne. If an mRNA analysis shows that exon 46 is absent, which would shift the reading frame, then skipping of exon 45 could restore it.”

**Will exon skipping be a therapy for my son?** Many Duchenne families from all over the world are asking me this question. To answer it: because exon skipping is a mutation-specific technique, you first have to know the exact mutation in the dystrophin gene of your sick boy, which can best be determined in a modern genetic laboratory with the MLPA method (multiplex ligation-dependent probe amplification), which analyzes all 79 exons in Duchenne boys and their mothers and other female relatives.

With the mutation known – deletion, duplication, or point mutation – you may examine the gene sequence of the 13,990 bases or genetic letters of the combined 79 exons of the dystrophin mRNA. You can see and download this sequence from the Leiden Muscular Dystrophy Pages on the internet: www.dmd.nl/seqs/murefDMD.html. From this sequence you can determine whether a particular mutation shifts or maintains the reading frame after the mutation, thus whether this genetic information predicts a Duchenne or Becker dystrophy for your child.

By looking at the border sequences of the exons, you can also determine which exon or exons must be skipped for bringing the reading frame from out-of-frame back to in-frame again. On the last page of this report, I am showing the molecular details of the skipping of exon 51 to restore the shifted reading frame caused by the deletion of exon 50. In a similar way, you can determine which exon your son needs to have skipped.

But you have to have experience to do this, so to facilitate this task, Annemieke Aartsma-Rus has included in her PhD thesis several lists from which you can read directly which exon or exons must be skipped when you know the details of your son’s mutation. These lists can be seen on the internet:

Deletions: www.humgen.nl/lab-aartsma-rus/Table%20deletions.pdf

Duplications: www.humgen.nl/lab-aartsma-rus/Table%20duplications.pdf

Point mutations: www.humgen.nl/lab-aartsma-rus/Table%20point%20mutations.pdf

Here is a selection of 10 entries in the list for deletions:

<table>
<thead>
<tr>
<th>Deleted exons</th>
<th>Exon(s) to skip</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 – 43</td>
<td>44</td>
</tr>
<tr>
<td>43 – 45</td>
<td>46</td>
</tr>
<tr>
<td>43 – 50</td>
<td>51</td>
</tr>
<tr>
<td>43 – 52</td>
<td>53</td>
</tr>
<tr>
<td>44 – 43 or 45</td>
<td>43+51</td>
</tr>
<tr>
<td>44 – 50</td>
<td>43+51</td>
</tr>
<tr>
<td>46 – 47</td>
<td>45</td>
</tr>
<tr>
<td>46 – 52</td>
<td>45+53 or 53+54</td>
</tr>
<tr>
<td>48 – 50</td>
<td>51</td>
</tr>
<tr>
<td>51 – 53</td>
<td>50</td>
</tr>
</tbody>
</table>

There is another rather easy way to find the exon or exons to be skipped if you know the mutation of the dystrophin gene – deletion, duplication, or premature stop. The arrangement of the 79 exons can be seen in the following picture which I have copied from one of Annemieke’s ppt-slides she showed at the 2009 PPMD annual meeting in Atlanta.

If in this representation the connection between two exons points to the left, the border of the two exons is between entire code words, if it points to the right, the border is between the first and the second letter of a code word, and if it is vertical, the border is between the second and the third letter of codon.

To find which exon to skip, cross out the deleted exon
or exons and see whether the exon before or after the deletion must be taken out, to be skipped, so that the remaining exons fit to each other in one of the three “natural” ways. For instance, a deletion of the 8 exons 45 – 52 means that the two exons 44 and 53, flanking the deletion, do not fit together. But skipping exon 53 would produce a normal fit between the end of exon 44 and the beginning of exon 54. You will also immediately see that the deletion of exons 44 – 50 could be repaired by skipping both flanking exons 43 and 51, so that exons 42 and 52 would fit to each other.

You will also realize that a deletion, e.g. 44, that produces non-fitting exon borders mean a shifted reading frame leading to Duchenne, while a deletion, e.g. 48 – 51, that produces fitting exons do not shift the reading frame, and that should mean Becker dystrophy. This procedure works also for duplications and premature stop codons.

Annemieke has explained how exon skipping and PTC124 works for different mutations on her website: www.humgen.nl/lab-aartsma-rus/index%20for%parents.

It is quite interesting to know that the dystrophin gene has mutation hot spots: 50% of all mutations involve deletions of one or more exons between 45 and 53 and 20% between the exons 2 and 20.

If with these lists and explanations you have found which exon or exons have to be skipped in the dystrophin mRNA of a Duchenne boy, it is important to understand that this does not guarantee that his severe Duchenne symptoms will be changed into the milder symptoms of Becker dystrophy, if he will be treated with his “personal” exon-skipping drug as shown. All they can say is that a particular skipping will change the reading frame of the genetic message on the mRNA from out-of-frame to in-frame again. It does not say that the in-frame genetic message will produce a “Becker”-dystrophin in every case, because the reading-frame rule has many exceptions, as has been explained in the recent publications by Terence Partridge (13) and Eric Hoffman (14).

The reasons for these exceptions are not completely understood in each case. For instance, the borders of the deletions in the dystrophin gene do not necessarily correspond to the borders of the exons but may lie somewhere inside the often very large introns between the exons. These deletion borders are normally not determined by the usual genetic test methods, and they may be different in patients with the same deletions. Because the introns contain sequences which are important for the regulation of genes, their presence or absence may produce different disease symptoms. On the other hand, the dystrophin protein has a structure with regions of different importance. Some deletions together with the skipped exons may, in some cases, produce an altered protein structure that does not allow the correct functioning of the shortened dystrophin.

Thus, an exon skipping therapy will in many cases produce a protein that reduces the dystrophic symptoms, but there might be surprises which will only become apparent during an actual treatment.

**Different types of potential exon skipping drugs.** The AOs used by the Dutch researchers were first abbreviated as AONs, because they are protected oligonucleotides. But the morpholinos are similar to, but not real nucleotides, so the abbreviation AO for antisense oligo is now used generally for all antisense compounds, also in this report.

For the animal experiments and the first exon skipping clinical trials, two kinds of chemically protected AOs are used. They have to be protected because then they are not or only slowly destroyed in the muscle cells by nucleic-acid-destroying enzymes.

The Dutch scientists are using 2’O-methyl-phosphorothioates, also called methyl thioates 2’O-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 the British researchers are using have one of the phosphate oxygens replaced by a dimethyl amide group, a nitrogen carrying two methyl groups, and the entire ribose units are replaced by morpholino rings, six-membered rings, each consisting of 4 carbon atoms, 1 oxygen and 1 nitrogen atom with hydrogen atoms attached to the carbons at the corners of the structures.
I am showing you here the chemical structure of these two types of AOs with just two of their genetic letters indicated. The 2’O-methyl AO used in the Dutch trials, PRO051, has 20 genetic letters with the sequence:

UCUUUACGUAGGAACUC

The morpholino AO used in the British trials, AVI-4658, has 30 letters, which include the 20 letters of the Dutch AO (underlined).

GAUCUUUACGUAGGAACUACAACCUC

Both of these AOs attach themselves to the exonic-splicing-enhancer sequence, ESE, because their sequences are complementary to the ESE sequence (and its surroundings) inside exon 51. This sequence is important for the normal splicing process, for the inclusion of this exon in the dystrophin mRNA. If it is blocked by the AO, the exon is skipped, excluded from the mRNA.

The molecules of these AOs have quite large and complicated chemical structures. The 2’O-methyl PRO051, for instance, consists of 699 atoms and the morpholino AVI-4658 of more than one thousand. This shows you that these potential drugs for our Duchenne boys are quite different from “normal” drugs, they are also difficult to make and they will be expensive.

The Dutch researchers have recently compared the 2’O-methyls with the morpholino AOs for skipping exon 23 in living mice. After local and systemic injections of the AOs, they found that there are indeed differences of efficiency, as described before, when mdx mice were treated. However these differences were less pronounced or even absent when AOs targeting human exons were injected in hDMD mice which contain normal human dystrophin genes. What one can learn from these preliminary studies is, that possibly some exons will be skipped more efficiently by morpholinos, others by 2’O-methyls and for some both may be equally efficient. However, these animal studies are only an indication of what may happen in human patients.

You should get to know some of the scientists who are working for our children. At this point, I think, it is time to show you pictures of a few of our scientists who, together with their co-workers, are engaged in finding an exon-skipping treatment, and whose work I am reporting here. Annemieke’s picture is the first, you have seen how often I mentioned her. We have written many e-mails to each other, she has helped me whenever I needed help, and always very fast within a few hours. My special thanks go to her.

Annemieke Aartsma-Rus PhD. University of Leiden

Three new types of AOs. Three more types of AOs – one with tails of a short chain of amino acids, a peptide, the second with a protein-like backbone, and the third with an added branched structure – have been developed which may be more effective than the two first ones now being tried in Duchenne patients:

Exon skipping with peptide-conjugated nucleic acids. One of the problems of the morpholino AOs is that they have difficulties entering all muscle types, especially the heart. To overcome this problem, Matthew Wood and his colleagues at the University of Oxford in collaboration with AVI BioPharma in Portland/Oregon modified the morpholino AO (PMO) for skipping exon 23 in mdx mice by attaching short amino-acid chains, peptides, to the antisense structure.

After experimenting with several different combinations of peptides, the researchers developed a peptide-conjugated PMO with two adjacent peptides attached to the usual morpholino AO of 25 base units against exon 23 of the mouse. The mdx mouse has a premature stop codon in its exon 23 which is the reason for the absence of dystrophin in its muscles. Skipping of this exon restores the production of dystrophin.

The first attached peptide is the B-peptide, previously developed by AVI Biopharma, which helps the AOs to cross cell membranes. The second peptide is the muscle specific peptide MSP, which was designed to lead the AO specifically to muscle tissue. The B-peptide consists of 14 amino acids and the MSP of 7 and they are joined together into a single peptide chain. I think you will be interested to see the complete 47 components of the structure of this exon skipping drug for dystrophic mice, written in the abbreviations used by the scientists.

RXRRBRXRRBRXBX – ASSLNIA-X – GGCACACCCTCGCTTACCTAAAT

Three weekly systemic injections of 6 mg/kg of this optimized AO into mdx mice restored widespread high-level dystrophin expression in skeletal as well as cardiac muscles, the reappearance of the dystrophin-associated protein
complex on the muscle cell membranes, a normalization of the CK activity, and a significant improvement of muscle function (16).

Just before finishing this update of my report, Aurélie Goyenvalle informed me that she and her team in the laboratory of Kay Davies in Oxford have used this kind of a peptide-conjugated morpholino AO to treat very severe dystrophic mice which not only are missing dystrophin but also the similar protein utrophin because their utrophin gene had been genetically destroyed. In contrast to the “normal” mdx mice, these double-KO mice have Duchenne-like symptoms. The treatment with the peptide-conjugated AO induced a near normal level of dystrophin expression in all skeletal muscles examined, resulting in a considerable improvement of their muscle function and dystrophic pathology. The average lifespan of these very dystrophic mice is usually around 8-9 weeks of age. The mice treated with the conjugated morpholino AOs are still alive after 7 months and look very healthy. After an initial treatment with 6 weekly injections, they were only re-injected once a month (26).

AVI Biopharma has developed this type of a peptide-conjugated AO against the human exon 50, called AVI-5038, in preparation of clinical studies in the United States.

Exon skipping with peptide nucleic acids. Another group of AOs, peptide nucleic acids, PNs, are now also being investigated for their exon skipping properties. Instead of the sugar-phosphate backbones of DNA and RNA, peptides form the backbone of PNs. They are water-soluble, very stable, can easily be modified and designed to carry the usual bases of DNA and RNA in the correct spatial arrangement with any desired sequence so that they can act like other AOs. Matthew Wood and colleagues in Oxford showed first that a PNA targeting exon 23 showed significant activity by intramuscular injection into mdx mice (28). More recently they showed that a 25-mer PNA has very good activity when injected systemically into mdx mice (29).

Matthew Wood is now working closely with Michael Gait and his colleagues at MRC Laboratory of Molecular Biology in Cambridge UK on peptides conjugated, meaning joined, to both the PNA and PMO against exon 23 of mdx mice. The team has developed a series of PNA-internationalization peptides, the initial candidate being Pip2b, which was joined to an anti-mouse-23 PNA of 20 units with the following abbreviated structure:

\[ \text{RAhxRRAhxRRAhxRIHILFQNdRMKWHKßAlaC} \]

The intramuscular injection of only 5 micrograms of this Pip2b-PNA-AO into 8-week old mdx mice showed a high number of dystrophin-containing fibers in the single injected mouse muscle (17). The most exciting development is that an improved version of the peptide, known as Pip5e, in conjugation with a PMO 25-mer, has now shown very high dystrophin production in all muscle types after a single 25 mg/kg intravenous injection into mdx mice, and for the first time includes heart muscle (Yin, Saleh, Gait, Wood, manuscript in preparation). To date it has been very hard to find peptides as PMO conjugates that have high activity in heart, but Pip5e, which is a shortened and simplified peptide compared to Pip2b, is a breakthrough in this respect and is likely to be evaluated further preclinically.

Exon skipping with octa-guanidine morpholinos. Qi Long Lu and his co-workers at the Carolinas Medical Center in Charlotte, North Carolina, have developed another modified AO which can cross the muscle cell membranes with very high efficiency and produce dystrophin in the heart.

To the normal morpholino AO, they added a complex branched chain structure (a polymer) whose eight branches carry a guanidine group (octa-guanidine) at their ends. These end groups are part of the amino acid arginine, which are components of the other two new AO types described here. This octa-guanidine polymer together with the morpholino is called vivo morpholino. Unlike peptide-linked morpholino (PPMO), the octa-guanidine polymer is not a peptide, it is therefore unlikely that the body’s immune system will respond to and destroy the compound.

With the vivo morpholino against exon 23 of mdx mice, experiments on living mice were performed. The systemic bi-weekly treatment during 10 weeks with intravenous injection of only 6 mg/kg AO produced up to 50% new dystrophin in all fibers of the skeletal muscles, in up to 10% of the cardiac muscles, and also in the slow muscles of the blood vessels and intestines. The muscle functions were improved significantly and there were no side effects and no immune rejection. These new AOs offer a realistic perspective for an effective Duchenne therapy (21).

Exon skipping with gene transfer.

First approach: The researchers at the Institut de Myologie in Paris, Luis Garcia and Aurélie Goyenvalle (now at the University of Oxford) and their co-workers are trying to combine exon skipping with gene therapy by instructing the muscle cells to produce the AOs themselves, so that they do not have to be injected repeatedly. This can be achieved by genetically instructing the muscles to produce modified U7-snRNAs containing the genetic information for the construction of the AOs. U7-snRNAs are small RNAs in the cell nucleus which have a structure similar to splicing factors.

The researchers constructed a modified gene for the U7-snRNA by adding the complementary DNA sequences for two AOs which are necessary for skipping exon 23 of mdx mice. These short snRNAs are also “made” by genes. This modified U7-gene, U7 SD23/BP22 together with control sequences, was placed into the DNA of type-2 adenovirus, AA.A. These transport vectors were then injected first locally into single muscles of mdx mice and then systemically into their blood circulation. In the muscle cells, the incoming DNA sequences were transcribed into the corresponding RNAs which then per-
formed the usual exon skipping process that restored the reading frame and thus opened the way for the synthesis of dystrophin without the amino acids determined by exon 23. This new shortened dystrophin appeared in up to 80% of the fibers of the treated muscles where it migrated to its normal position underneath the cell membranes and was stable for more than one year without causing any immune reaction.

The dystrophic processes in the mdx muscles, that is, their accelerated degeneration and regeneration, were completely halted. The systemically treated mdx-mice, which were physically stressed by running in a treadmill, did not develop the usual muscle damage found in non-treated mdx-mice (19).

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

Second approach. More recently, Aurélie Goyenvalle developed a new, more general technique, in Kay Davies’s laboratory in Oxford. In this approach, the exon skipping is mediated by a new and almost “universal” U7snRNA vector that is bifunctional, because it carries a complementary DNA sequence to the exon to be skipped and also a free tail which has binding sites for the heterogenous nuclear ribonucleoproteins A1/A2 (hnRNP). These proteins can, when they come near the spliceosome at the borders of an exon, inhibit the splicing process of that particular exon so that it is not included in the final pre-mRNA.

The complementary DNA sequences of the bifunctional U7 gene brought by the viruses, once they are transcribed into small RNAs, will attach to the exon to be skipped, and attract the hnRNP's close to the splicing sites. They will therefore induce the skipping of the targeted exon by repressing the normal splicing process. Thus, this kind of exon skipping is not brought about by the usual AOs but by these “universal” proteins that are the same for inhibiting the splicing of all exons.

The reason for this approach is to shorten considerably the lengthy optimization process usually required for every new exon targeted. As with the normal skipping approaches engineering the best possible AO structure for skipping a new exon is a long procedure that can now be shortened by the use of the “universal” tail.

This method has already been tried successfully in the laboratory for skipping exon 51 in isolated myoblasts from a Duchenne patient with a deletion of exons 49 and 50. The most effective U7snRNA was the one with the tail structure called A1 (U7ex51-AON-A1). It restored the dystrophin level to almost normal in these myoblasts. By injecting this bifunctional structure locally into muscles of the “humanized” mdx mouse developed by the Dutch researchers which contains human dystrophin, an exon skipping rate of 54% was reached.

It is expected that this technique will also be able to skip other exons and even those which are difficult to skip by “normal” exon skipping. This vectorized approach also offers the possibility to perform multi-exon skipping by using two or more U7snRNAs.

This use of the bifunctional U7snRNA has been published in the journal Molecular Therapy with the title: “Enhanced exon-skipping induced by U7snRNA carrying a splicing silencer sequence: promising tool for DMD therapy” (25).

Genetic U1 exon skipping, Irene Bozzone and her coworkers at the University La Sapienza in Rome have pioneered a genetic exon-skipping method in which they are using the gene encoding for the U1 snRNA, a small nuclear RNA involved in the recognition of the 5'-splice site and required for the first step of the splicing reaction.

They have substituted the first 9 nucleotides at the 5'-terminus of the U1 snRNA with 54 nucleotides complementary to the border regions of exon 23 of the mouse dystrophin gene. The entire structure was then inserted into adeno-associated viruses of type 2/1. Three to 4 trillion (3-4 x 10^12) of these virus vectors were injected into the tail vein of 6-week old mdx mice. Twelve weeks afterwards, correctly skipped mRNA and also new dystrophin in 10-20% of the normal amount could be detected in several skeletal muscles and also in the muscles of the heart and the diaphragm. The mice also had significantly improved muscle functions.

In another experiment, 18 months after the one-time injection of the viruses, the muscles of two mice were examined when they were 80 weeks old. During this entire time, practically until their natural death, the skeletal, cardiac, and diaphragm muscles of these old mice contained not only the corrected, in-frame, mRNA without exon 23 and sufficient slightly shortened dystrophin, which maintained the normal degeneration-regeneration process, but the muscle functions were also improved significantly without any immune problems. These results show that this genetic technique may lead to a long-lasting treatment of Duchenne patients.

Recently, the U1-based exon skipping technology has been licensed to the Dutch company Amsterdam Molecular Therapeutics (AMT) that is proceeding towards pre-clinical and clinical studies.
Personalized therapies and the regulation agencies.

The Federal Drug Administration, FDA, in the United States, the European Medicines Agency, EMEA, and other regulation agencies require that the “normal” development of a classical drug has to go through the following stages:

The pre-clinical phase involving laboratory and animal studies to assess its safety, biological activity, and formulations;

- the clinical phase-I trial on 20 – 100 healthy volunteers to determine its safety in humans;
- the clinical phase-II trial on 100 – 500 patients to evaluate optimal dosages, safety, and efficacy; and
- the clinical phase-III investigation on 1,000 – 5,000 patients to confirm the drug’s safety and effectiveness of long-term use.

The cost of all three clinical investigational trials can be up to 500 million US$ for one drug and it may take up to 15 years from the first concept of a new drug until its market approval.

The regulatory rules were made in an age when patient-specific types of approaches as exon skipping were not yet available. To get a personalized Duchenne drug through these stages, several challenges are encountered which are new to the agencies and which have to be overcome before they can reach the patients.

For instance, phase-I safety studies with AOs in healthy volunteers could shift the reading frame in a normal dystrophin mRNA and, if they are sufficiently effective, give the healthy volunteers Duchenne dystrophy, an unwanted side effect which has nothing to do with “normal” adverse events. Another major concern would be so-called off-target effects, meaning that exons could be skipped in other genes than the dystrophin gene.

Another problem, not encountered in normal drug development might be specific to Duchenne dystrophy: because of the absence of dystrophin, the cell membranes of Duchenne muscles have tears and holes through which the exon skipping drugs can get into the cytoplasm of the cells and also into the nucleus where they are needed for their therapeutic action. Thus, certain adverse effects might appear only in patients but not in people without Duchenne. This makes the standard safety studies with healthy volunteers problematic.

The team of Eric Hoffman at the Children’s Medical Center in Washington DC has recently received 2.5 million US dollars from the US Department of Defence for studying in mice and monkeys these toxicity concerns of the AVI anti exon-51 AO used in the British clinical trials.

Another challenge for the approval of exon skipping drugs would be the cost of several hundreds of millions of dollars and the time necessary for going through all four stages with each of the many different AO sequences required to treat patients with different mutations. Most of these individual sequences will be applicable only to a small number of patients, thus, in many cases, there will just not be a sufficient number of patients available for the standard clinical studies.

If the ongoing first exon skipping trials continue to show no drug related adverse effects, as it seems to be likely, the regulating agencies can hopefully be convinced to consider entire groups of AOs of a certain chemical type as one single drug and thus shorten considerably the approval process.

First things should come first however. Although the progress in exon skipping seems promising, the road to eventual access for patients to a safe and efficacious treatment is still a long one. Once the first drug, aimed at exon 51, has been developed successfully and has been approved by the regulatory authorities, the question as to how other exon skipping agents can be developed more efficiently, becomes important. At the same time, all efforts should be focused on helping as many patients globally as possible and as quickly as possible, without jeopardizing the safety of the boys and without taking undue shortcuts.

At a first workshop, organized by TREAT-NMD, to address this topic, a large group of 98 Duchenne experts, including the scientists involved in the current clinical exon-skipping trials, met on 25 September 2009 at the London offices of EMEA, the European Medicines Agency, to begin a dialogue on the regulatory issues surrounding the unprecedented level of personalization for the therapies for genetic conditions like Duchenne dystrophy. The meeting with EMEA sought to take steps to identify a pathway that will allow the safe and efficient progress of these drugs through the approval process within an acceptable time period.

Part of this summary is based on a new publication by Eric Hoffman of the Children’s Medical Center in Washington DC (13) and a press release by TREAT-NMD on the 2nd of October.

Clinical trials

Four clinical trials for skipping exon 51 in Duchenne boys are described in this most important section of this report. Two of them are local ones, in which only one unimportant muscle is treated. These treatments of a single muscle cannot improve the function of all muscles, they cannot provide a clinical benefit to the participating Duchenne boys. The two other trials are systemic ones, in which the potential drugs, the AOs, are injected into the blood circulation. They may already show a small improvement of the muscle function.

But the main question these four trials are designed to answer is: Are the potential new drugs safe? After all, they may have to be given for many years during the hopefully extended lifetime of the boys.

These indispensable steps to the full development of a therapy are just human experiments which also can go wrong. Participation of a sufficient number of boys who need exon-51 skipping is important, but it is not worthwhile for their families to undertake expensive journeys to the trial centers. Kate Bushby has explained this in the in-
“I don’t think that it is practical for children to come from far away to participate in clinical trials, because they have to be at the clinical centers every week for injections. They have to have lots of blood samples taken. The families have to live near the centers. Families have to put their whole life on hold, even if they live close by. The families should realize that the trials are only trials. In the later efficacy trials, their child could even be on placebo. It may not help them and there may be unforeseen side effects. Trials are really hard work.

I would even say that the children in the trials are almost at a disadvantage. Because they have to go through a hassle to get to something everybody else will profit from later provided it is proven to work. We are very grateful indeed to the families and boys who take all the time and effort to take part in these studies, which we really hope will move things forward for everyone.”

You can see the entire interview with Kate Bushby and also the one with Hans Schikan and Judith van Deutekom on my internet pages: www.duchenne-information.eu.

Four Dutch boys participated in this open study. They were between 10 and 13 years old and had proven deletions of the dystrophin exon(s) 50, 52, 48-50, or 49-50. They were treated sequentially, meaning that only after the results for one boy were positive and did not show any serious side effects, the next boy was treated. Each boy received a single dose of 0.8 mg PRO051 dissolved in saline, 0.9% salt solution, under local anesthesia into a small region of approximately 1.5 cm length of his shin muscle.

After 4 weeks, muscle tissue was obtained in a biopsy from the injection site and tested for the expected skipped mRNA and novel dystrophin protein. Almost all muscle fibers in the biopsy tissue, up to 94%, showed novel dystrophin expression at the muscle fiber membranes in levels of 33%, 35%, 17%, and 25% compared to healthy muscle tissue. The treated muscle tissue volume was too small to provide any clinical benefit to the participating boys. This was expected and the boys and their families knew this.

These results showed for the first time, that exon skipping does not only work in mice and dogs, but also in human muscle, in Duchenne boys, and they signify also that an exon skipping treatment, when it becomes available, should be started when most of the muscles are still intact, that is, immediately after diagnosis and the exact determination of the mutation in the dystrophin gene.

The results of this first application of the exon-skipping technique in Duchenne boys have been published on the 27th of December 2007 in the New England Journal of Medicine with a commentary by Eric Hoffman (18).

Systemic clinical trial in the Netherlands with PRO051 to skip exon 51 in Duchenne boys. The results of a local intramuscular clinical study with PRO051 which was published in the New England Journal of Medicine in 2007 had shown that the exon skipping technique with this agent does not only work in laboratory animals, but is also able to produce new dystrophin without side effects in Duchenne boys. With the next step towards the full development of this technique, the scientists had to show that PRO051 can also be applied systematically via a subcutaneous injection (i.e. under the skin) so that the drug can reach and treat all muscle cells. The first systemic study ever has now been performed with PRO051.

Between July 2008 and January 2009, Prosensa Therapeutics BV did a systemic trial with twelve 5-15-year old Duchenne boys with PRO051 injected subcutaneously. Subcutaneous injections have the advantage that they would not require frequent visits to doctor’s offices and hospitals if repeated treatments will later become necessary. The injections and the medical supervision were done by the teams of Nathalie M. Goemans at the Department of Pediatric Neurology of the University of Leuven in Belgium and of Mar Tulinius at the Queen Silvia Children’s Hospital in Gothenburg in Sweden.

The results of the study were presented by Natalie Goemans on the 12th of September 2009 at the International Congress of the World Muscle Society in Geneva.

Each boy received one injection per week for five consecutive weeks. In this dose-escalation study, the first three boys received PRO051 at a dose of 0.5 mg/kg at each of the five injections, the next group 2 mg/kg, the third group 4 mg/kg, and the last three boys 6 mg/kg.
Muscle biopsies were taken before and two weeks after the treatment for the two first patients, and at two and seven weeks after the last injection for all other boys. The structure of the mRNA and the presence of new dystrophin protein were determined in all biopsy samples.

These analyses showed for the first time that the subcutaneous administration of PRO051 resulted in the specific skipping of exon 51 and induced the production of new dystrophin in a dose-related manner, which means that the highest drug dose created the highest amount of new dystrophin.

This first systemic trial with an exon skipping agent in Duchenne was designed to answer the most important question: is this genetic treatment safe? The answer was positive: no immune rejection of the new protein and no other clinically significant problems were found, thus this first whole-body therapy with an exon-skipping drug was well tolerated by all 12 Duchenne patients in the study. This is also illustrated by the fact that all 12 boys have entered the open label extension study and as per the end of September all boys have entered their tenth week of treatment.

The positive outcome of this trial encouraged all the involved scientists and clinicians to begin the preparation of the next large phase III trial with PRO051 which is expected to start early 2010 with more than 150 Duchenne boys who need skipping of exon 51.

Using the TREAT-NMD global register for Duchenne dystrophy, Prosensa has already identified about 300 patients in 21 countries who meet the inclusion criteria for this trial. These patients have been matched to 50 potential clinical centers. The selected patients and clinical centers will be contacted for participation in this large international trial.

The key question of this study will be whether the appearance of new dystrophin will also lead to an improvement of muscle function, for which many biochemical and clinical tests will be performed. It is expected that among these tests will be the standardized 6-minute-walk test to determine the distance in meters a still ambulatory boy can walk in 6 minutes.

**First local clinical trial in the United Kingdom.** Another clinical exon-skipping trial was performed in the UK between the autumn 2007 and the end of 2008 by the MDEX Consortium under the direction of Francesco Muntoni of the Imperial College London, and Kate Bushby of the University of Newcastle upon Tyne. The MDEX consortium, funded by the department of Health, groups together nine researchers as well as the charities Muscular Dystrophy Campaign, ActionDuchenne, and Duchenne Parents Support Group.

Eight different morpholino AOs were tested in cultures of normal and Duchenne human muscles and in living non-dystrophic mice which contained human dystrophin in their muscles by the Dutch and British research teams. The best results were obtained with the morpholino AO H51A developed by Steve Wilton in Perth (Australia) and shown by Dominic Wells in London to be sufficiently stable for a long-term clinical treatment. This morpholino AO is synthesized by the company AVI BioPharma Inc. in Portland, Oregon and is called AVI-4658 (20).

This trial was a local study to assess the safety and biochemical efficacy of this morpholino exon-51-skipping drug after one-time injections into a small and unimportant muscle, the extensor digitorum brevis (EDB) muscle at the outside of the foot. Seven 11-16-year old Duchenne boys, some of them wheelchair-bound, participated. They had mutations that needed the skipping of exon 51 to restore the reading frame, two boys with deletion of exons 48-50, two with deletion of exons 45-50, one with deletion of exons 49-50, one with deletion of exon 50, and one with a small deletion in intron 49 causing the deletion of exon 50. Before the treatment was started, cells from a skin biopsy were tested in culture to confirm that AVI-4658 indeed removes exon 51 from the dystrophin mRNA of each boy and produces the expected shortened dystrophin mRNA and protein in these laboratory experiments.

![Prof. Kate Bushby](image1.png)  
Prof. Kate Bushby  
University Newcastle u.T.

![Prof. Francesco Muntoni](image2.png)  
Prof. Francesco Muntoni  
Imperial College London

Each of the selected patients had less than 5% of revertant fibers in his muscles as determined by a biopsy at the time of their diagnosis. Different low amounts of these dystrophin-positive muscle fibers are present in many Duchenne boys. They are caused by spontaneous exon skipping. The amount of this revertant dystrophin found in the placebo control had to be deducted from the amount of dystrophin detected after the treatment.

Many other clinical investigations were also done to assure that the results were scientifically sound and caused by the treatment alone. For instance, the investigators who analyzed the muscle samples did not know whether the tissues came from the AO-treated muscle or the control muscle from the other foot, meaning that the trial was “single-blind”.

As this was a dose-escalation study, the first two boys received a low dosage of 0.09 mg of the morpholino AO in 0.9 ml saline, delivered with nine parallel injections of 0.01 ml each directly into the EDB muscle. The muscle of the other foot received similar injections of only salt solution for the control of the dystrophin background. The five other boys received a 10-times larger dose of 0.90 mg AO in a similar injection schedule. Almost the entire treated EDB muscles were removed in biopsies between three and four weeks after the injections.

The higher-dose AO resulted in increased dystrophin production in all treated muscles. In the muscle tissue
around the needle-track through which the drug was given, 44.79% of the muscle fibers had increased amounts of dystrophin. The mean intensity of dystrophin in several sections of the entire biopsy material ranged from 22% to 32% of the mean intensity in muscles of healthy persons, and it was 11-21% greater than the background of the revertant dystrophin in the control muscle from the other foot. In some single dystrophin-positive fibers, the amount of dystrophin was found to be up to 42% of that in healthy muscle. Additional tests proved that the new dystrophin had indeed the reduced molecular weight of the expected shortened Becker-type dystrophin.

The aim of this study was to show the local safety and biochemical efficacy of injections into a single muscle of a morpholino AO. As with all local treatments of this kind, no therapeutic benefit for the participating boys was expected by the treatment of the single small EDB muscle in one foot and there was no attempt to determine the function of the muscle before and after the treatment. The boys and their families were aware of this limitation.

This in-vivo study showed that the antisense drug AVI-4658 induced the intended skipping of exon 51 and the production of new dystrophin that had moved to its normal place at the inside of the muscle cell membrane and then correctly connected there to the proteins of the dystrophin-glycoprotein complex. This treatment was not associated with any systemic or local adverse side effects or with any immune response against dystrophin.

In the muscle tissue of the patients who received the very low dose of the drug, exon-skipped mRNA was detected, but no increase of the dystrophin protein over the background level could be proven, because the protein test method “western blot” was not sensitive enough to detect very small protein differences.

Indeed the case. The main technical difference between the two studies was that the Dutch scientists were only allowed to perform one single biopsy on the treated muscle after the treatment whereas the British could biopsy both, the treated muscle and the control muscle, after the treatment. Thus, in the Dutch study the dystrophin background could not be taken into account for the determination of the dystrophin content after the treatment. There were more differences between the two studies, meaning that the results of the two studies are not directly comparable in all details.

In the publication of the British results, published online on the 26th of August 2009 by Maria Kinali, Virginia Arechavala-Gomeza, Francesco Muntoni and 23 other scientists (23), the authors say: “Both studies reported unequivocal expression of dystrophin in similar concentrations”. Annemieke Aartsma-Rus and Gertjan B. van Ommen (24) state in their comment published at the same time, that “the next step which both groups are currently undertaking, is to deliver the antisense oligonucleotide systemically, therefore, detailed comparisons of intramuscular results, that is on single muscles, are largely irrelevant. Only systemic trials will reveal the true promise of this approach, and further trials are needed to validate the functional benefit, or at least the decline in disease progression.”

First systemic clinical trial in the United Kingdom.
One of the most decisive pre-clinical animal experiments for the preparation of this trial were 7 weekly AO injections into the tail vein of mdx mice which resulted in more than 50% of shortened dystrophin in most of their muscles compared to the normal amount. This new dystrophin was then present for at least 14 weeks (27).

After approval was obtained from the three UK supervising agencies MHRA, GTAC, and GOSH between August and November 2008, the first two boys received their intravenous injections of the AVI-4658 AO into the blood circulation at the beginning of 2009. In total, 12 still ambulant Duchenne boys in 4 groups who all needed skipping of exon 51 have now received their injections weekly for 12 weeks. The injections were done sequentially so that a later treatment can be stopped immediately if something happens in an earlier one.

This trial is again a dose escalating study, where the first boys receive a very low dose of 0.5 mg/kg of AO every week, and raised to 4.0 mg/kg for the boys treated in the fourth group. For a 10-year old boy in the fourth group weighing 30 kg, this meant that he received 1.44 grams of the AO drug during the entire trial. In earlier clinical trials for other diseases, AO doses of up to 300 mg/kg/day were well tolerated. The aims of the trial are to test for safety and tolerability and also to changes of muscle function and strength. Approval was obtained from the regulatory bodies to add 2 further groups of 4 boys each to receive 10 and 20 mg/kg of AO. Treatment of the fifth group is ongoing.

Although biopsies on several muscles would be necessary to prove that the AO drug injected into the blood stream has reached several muscles and produced dystrophin there, only one biopsy after the trial has been allowed by the regulation agencies. This biopsy was taken from the biceps of the boys.
This trial is being performed at the Great Ormond Street Hospital in London and the Royal Infirmary in Newcastle upon Tyne. Stephen B. Shrewsbury, Chief Medical Officer of AVI BioPharma, reported these preliminary results on September 12 at the meeting of the World Muscle Society WMS in Geneva. He mentioned that AVI-4658 was well tolerated by the patients already treated and that no drug-related side effects or other problems were detected. He concluded that “the encouraging safety data is extremely important for the future use of this antisense drug as a dose-limiting toxicity could severely limit the effectiveness of this Duchenne therapy in this chronic disease where treatment must start in childhood and probably continue for life”.

As soon as the details and the full results of this study are published within a few months, they will be included in the next update of this report.

AVI BioPharma is the sponsor of this trial, and Francesco Muntoni has been awarded 1.3 million US$ from the Medical Research Council of the UK to offset some of the costs of the trial.

International cooperation ICE. The organization International Collaborative Effort for DMD, ICE, is a partnership of 12 institutions in France, Italy, the United Kingdom and the USA, which under the direction of Jean-Claude Kaplan (France), Terry Partridge (USA), Adrian Thrasher (UK), Luis García (France) and Berch Griggs (USA) will develop details of joint genetic exon –skipping methods. ICE was founded in 2008 and is financed by the Duchenne Parent Project France and the Association Monégasque contre les Myopathies with 1.7 million euros. Fourteen of the 105 scientists and clinicians who belong to ICE met on the 6th of June 2009 in Monaco to discuss their results obtained in the first year of their collaboration.

Some personal final words.

Dear friends everywhere: As I said at the beginning, I wrote this research report and my earlier ones especially for you, the boys and young men with Duchenne muscular dystrophy and your families, so that you understand better what is being done to stop this disease. I hope my words were not too complicated for you, the young men and boys with Duchenne dystrophy and for your families. But I know that because you wish to know what the researchers are doing for you, you will have already some basic knowledge about the many scientific facts mentioned in this report. If you have problems you have perhaps friends who studied medicine or biology or others who can help you. Or you may ask me by writing in English, French, German, Spanish, Italian, I will answer all e-mails or letters, as well as I can, but not always immediately and only in English or German, I cannot write in the other languages without too many mistakes.

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

When once talking to Annemieke Aartsma-Rus, she said “Duchenne dystrophy helps us to heal itself”. So I told her that I will spread her words to the rest of our Duchenne community. She then said that she really stole this quote from Gert-Jan van Ommen, who originally said: “This disease wants to be cured”. The disease really is like that, because the Duchenne muscle cell membranes have tears and holes which allow the AOs to get easily into the cells to do their healing job there. To get them into normal muscles is much more difficult.

Mutation diagnostics. As you have seen, exon skipping is a mutation specific technique. That means the exact mutation must be known for the patient to later benefit from such a treatment. The MLPA method is now a widely used technique for detecting deletions and duplications not only in Duchenne patients but also in female carriers. Even if the mutation of the patient in a family is not known or cannot be determined any more, the MLPA method finds the dystrophin deletions and duplications in the mothers or other women related to them. This is important for genetic counseling which can avoid the birth of additional Duchenne boys in the extended family of a patient. But if a woman at risk can be assured that she is not a carrier, this can encourage her to have healthy children without fear of a recurrence.

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

Beware of miracle drugs and treatments. A treatment that is safe and effective for a long time can only be developed with strictly scientific methods. If you see “miracle” drugs on the internet or get offers of miracle treatments, which cost thousands of dollars or euros and you consider getting and applying them for your child, please ask the miracle providers how many Duchenne boys they have al-
Recognized for my report writing. The president of Germany, Horst Köhler, has awarded me the “Bundesverdienstkreuz”, the medal of merit of our country for writing my reports, and in a quite impressive ceremony on the 5th of February 2009 it was given to me by state secretary Gündolf Fleischer in our Black Forest village Breitnau, where I live. I used this opportunity to explain to the about 120 persons present the situation of the families with Duchenne children in Germany and then started a correspondence with 12 high-level politicians including our Federal President and his wife Eva Luise Köhler, who takes care of rare diseases, and the Federal Ministers for Health and Research. During the coming winter months, after our new government will be in place and working, I will use these and new contacts with the aim to find ways to alleviate at least partially the large financial problems that the international Duchenne research community faces in its efforts to develop all the “beyond-51” exon-skipping drugs for all Duchenne boys who need them.

And on the 21st of March, the Gaetano-Conte-Academy of Naples in Italy awarded me its 2009 Prize for Social Research at the meeting of the Mediterranean Society of Myology in Nicosia on Cyprus. At that occasion, I presented a paper on how I explain exon-51 skipping to the Duchenne patients and families. If you wish to see the 55 powerpoint slides of my presentation, please ask me per e-mail, and I will send them to you. They are quite detailed and have many animations, so you will understand them without an explanation by me.

As a “thank you” for this award, I have written a publication about my work with the title “The Progress Reports on the Development of Therapies of Duchenne Muscular Dystrophy”, which will appear with an editorial by Reinhardt Rüdel in the October-2009 issue of the journal Acta Myologica.

Why must my son have this terrible disease? This is a question which I often hear when I receive e-mails and phone calls from almost everywhere in the world, most often from the mothers of Duchenne children. Although I have not learnt professionally how to deal with this question, I am trying to answer it anyway, in my own words like these:

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

The mutations that cause Duchenne muscular dystrophy do not punish you, the sick boys, or your mothers, who may have passed on the damaged gene to you. The mutations just happen, probably most often by mistakes when the gene is duplicated during cell division.

This is not the place to discuss religious questions which easily come to mind. Just allow me to add one thought: Nature seems to act blindly without regard to whom she hurts, on the other hand, a long series of her good mutations gave us the human brain, the most complex structure of the universe, which is able to solve many problems including how to repair these accidents causing Duchenne dystrophy. This report shows you that this is exactly what is happening!

I am sending all of you my best regards from my summer working place in Cadaqués on the northern end of the Costa Brava in Spain.

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The 3rd of October 2009

This report has illustrations, which will help you to better understand my explanations. It also contains pictures of some of the scientists who are working on finding an exon-skipping therapy for “our” boys. This personal touch, first offered in my original report on exon skipping, has been well received by the families and all the other readers, too. And here is a photo of a very special lady together with three children. This is Pat Furlong, the president of the American Parent Project Muscular Dystrophy, PPMD, on a picture taken by me at the PPMD meeting 2008 in Philadelphia. Many of you know her personally, and most know what she did and is still doing for accelerating research for a Duchenne therapy at an ever increasing speed toward an effective therapy to be ready, hopefully, within a few more years. We cannot be thankful enough for what she is doing!
References

If you wish to read one or the other of the original publications, go to [www.ncbi.nlm.nih.gov/sites/entrez](http://www.ncbi.nlm.nih.gov/sites/entrez) and enter the names of one or two authors into the "search" space in lower-case letters, like, e.g.: van deutekom jct. You will get the abstract for free and in many cases also the entire paper. To download the newer ones, there will often be a charge of about 10 to 20 dollars. You may also ask me, I can send you some of the papers as pdf-files.


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Thank you!

I am thanking all those whom I asked to help me to write this report by looking through my summaries of their work and making changes and additions where it was necessary: Annemieke Aartsma-Rus, Aurélie Goyenvalle, Judith van Deutekom, Kate Bushby, Terry Partridge, Francesco Muntoni, Irene Buzzoni, Qi Long Lu, Michael Gait, and Hans Schikan. I am thanking also TREAT-NMD and PPMD for financial support.

This is Ricardo Rojas in Mazatlan in Mexico who translated my first research report in 2000 into Spanish and then all the 12 others, too. You can see his translations of my recent reports and interviews on my internet pages. He will also translate this one.

He knows chemistry, biochemistry, molecular genetics and a lot of medicine which allows him to change all my scientific words into the correct Spanish ones. And he knows also what it means to have muscular dystrophy, because he has one himself: Becker dystrophy. We are all very thankful for his efforts to let all the Spanish speaking families know the results of Duchenne research.

Many of my reports have been translated also into other languages like French, Italian, Slovakian, Dutch, Polish, Czech, Greek, Turkmen, Japanese, Chinese, and probably into others I don’t know about yet.

As one of my many translators, I am showing you Yumiko Yamauchi who is a graduate student at the Department of Pediatrics of Kobe University in Japan. Her translation of my last exon-skipping report is very beautiful and makes me feel like an illiterate. Thanks to all of you!

Internet and mailing lists. You can see this updated report on the internet at www.duchenne-information.eu as well as recent interviews and earlier reports. If you wish to receive all my future reports as soon as they are ready, please send me your e-mail address for inclusion in my English, German, or Spanish mailing lists which already contain more than one thousand addresses.
Molecular details of skipping exon 51.

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

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

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

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

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

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