

## Research approaches for a Therapy of Duchenne Muscular Dystrophy.

### Part 1: Exon Skipping.

Published on the 30<sup>th</sup> of April 2009.

This report on *exon skipping*, the most advanced genetic technique for an effective therapy of Duchenne muscular dystrophy, is the first of several specialized texts, 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. The following parts of the entire research report 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 a number of other pharmacological possibilities as well as the current diagnostic 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 not a medical doctor, my reports contain only information on therapeutic research but not on the medical treatment and management of Duchenne patients.

I have written this text as all my earlier reports first in English and am translating them into German afterwards. A few weeks later, they will also be available in Spanish, translated by *Ricardo Rojas* in Mexico who has Becker

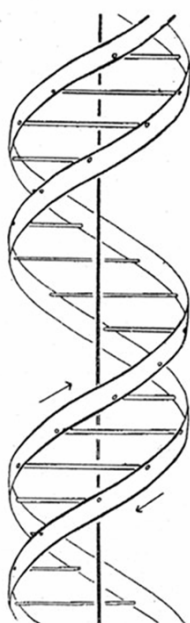
dystrophy. As before, all my reports and this one too, are not scientific publications with many difficult words, because I have tried to write them in a way that will let you understand what is happening for you in the laboratories even if you have not studied modern biochemistry and genetics.

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.

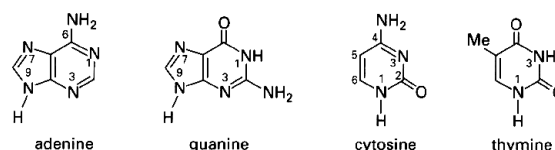
If you have questions concerning exon skipping and other Duchenne research, please send an e-mail to my German address [gscheuerbrandt@t-online.de](mailto:gscheuerbrandt@t-online.de) in English, French, German, Spanish, or Italian. I will try to answer all of them, but only in English or German.

### How genes make proteins

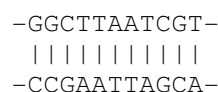


**Genes** are functional units of the genetic material **deoxyribonucleic acid, DNA**. Its structure looks like an intertwined ladder, the *double helix*, which was described in 1953 by *James Watson* and *Frances Crick*.

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. This is the structure of the four bases:



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:

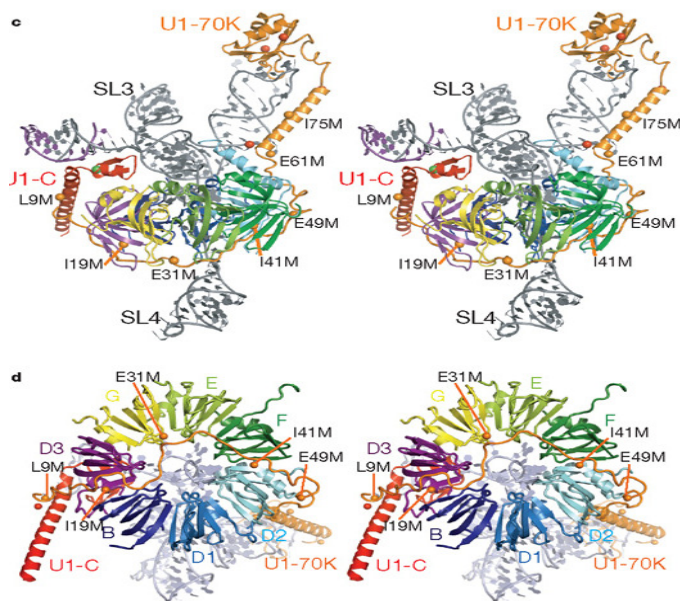


This sequence of the bases, of the *genetic letters*, is the **genetic information** for the development and maintenance of a living organism, and it is passed on from one generation to the next.

Most of the genes carry the instructions for the bio-synthesis 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 the often much longer **introns**, which do not contain only “genetic junk”, as one once thought, but also important information for the control of gene activities. 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 *ribosomes*, the protein synthesizing structures, in 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 recently published in the

26-March-2009 issue of 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 9).

**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.

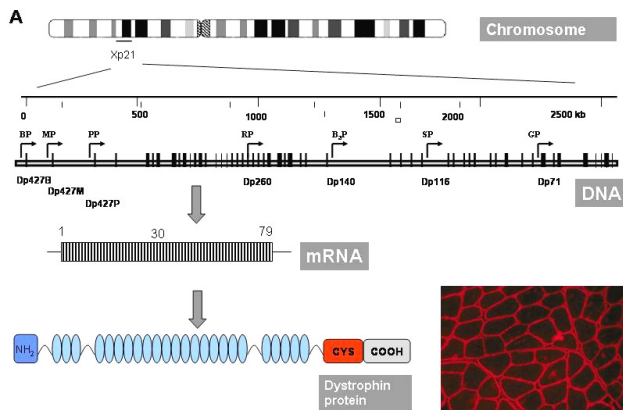
You can watch a film showing a gene making RNA and the ribosome assembling a protein by using the information in the RNA as fast as it happens in nature at: [www.youtube.com/watch?v=D3fOXt4MrOM&feature=related](http://www.youtube.com/watch?v=D3fOXt4MrOM&feature=related) – click on this address + Strg 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.

## Dystrophin gene and protein.

**Duchenne muscular dystrophy** affects only boys – about each 3,500<sup>th</sup> newborns – 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.



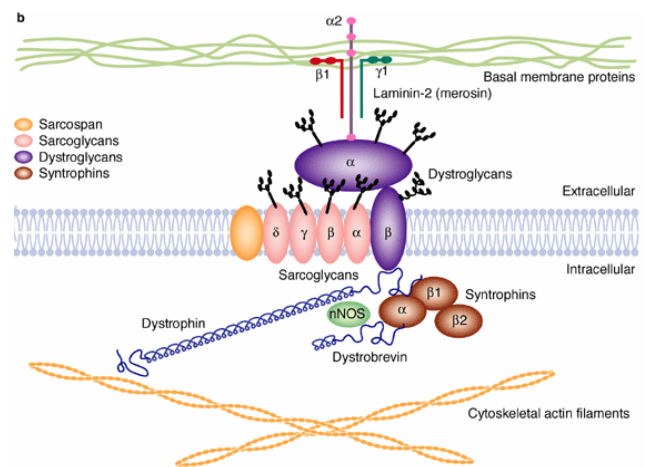
The dystrophin protein has a rod like shape with 24 repeated amino acid sequences separated by 4 hinge regions. Its two end regions are called the N and C terminals, and there is also a region with many cysteins, sulfur-containing amino acids. In the right corner is a cross section of muscle tissue with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

### The dystrophin-glycoprotein complex.



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 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 consequences, among them the reduced ability of blood vessels to relax, when blood flow increases, leading to respiratory and other problems, and also the gastrointestinal tract is affected when the motility of the intestines is reduced. So the damage of just one gene can affect 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 quadrillion ( $1.5 \times 10^{15}$ ) dystrophin molecules. A 5-year old Duchenne 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 biosynthesis of the protein. In order to make his remaining muscles 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 ( $200 \times 10^{12}$ ) 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 discussion in the mid-90s, *Gertjan van Ommen* of the University 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 procedure is not only being tested on sick mice and dogs but already on Duchenne patients.

Local and systemic clinical trials – on one single muscle or on all of them – for skipping exon 51 of the pre-mRNA in Duchenne boys have been and are being performed in Leiden, Leuven, and Göteborg with the help and partial funding by the Dutch company **Prosensa** 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 Netherlands 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 genetic 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 protect to a certain extent the muscle cell membranes. Therefore, the symptoms of the disease will be milder, the muscle degeneration will proceed more slowly, and the life expectancy 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 replaced nor repaired, but only the mechanism of reading its information 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 published 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 Aartsma-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 11<sup>th</sup> 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:

Rank	Exon to be skipped	% of all patients
1	51	13.0
2	45	8.1
3	53	7.7
4	44	6.2
5	46	4.3
6	52	4.1
7	50	4.0
8	43	3.8
1 – 8		51.2%

You can see the entire list on the internet at the address: <http://www3.interscience.wiley.com/journal/121645168/abstract?CRETRY=1&SRETRY=0>, it has also been published in March 2009 (7).

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 Prosenza 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 duplicated 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 – 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](http://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 have been published at Annemieke’s website [www.dmd.nl/gt](http://www.dmd.nl/gt), then go to “applicability”. Here is a selection of 10 entries in the list for deletions:

Deleted exons	Exon(s) to skip
40 – 43	44
43 – 45	46
43 – 50	51
43 – 52	53
44	43 or 45
44 – 50	43+51
46 – 47	45
46 – 52	45+53 or 53+54
48 – 50	51
51 – 53	50

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.

It is important to understand that these lists do *not guarantee* that the severe Duchenne symptoms of a Duchenne boy 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).

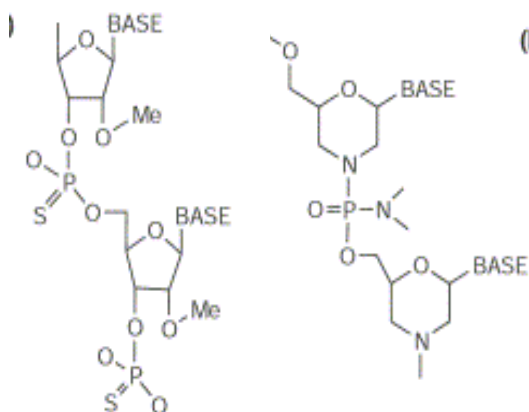
The reasons for these exceptions are not completely understood in each case. For instance, the borders of the deletions in the dystrophin gene do not necessarily correspond to the borders of the exons but may lie somewhere inside the often very large introns between the exons. These deletion borders 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* or *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.



2'O-methyl AO

morpholino AO

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:

UCUUUACGGUAGAAGGAACU

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

GAUCUUUACGGUAGAAGGAACUACAACCUC

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 (15). 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, as discussed in my interview with *Kate Bushby* on pages 13-15.



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**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 a picture of the first of four 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 have 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!

**Two new types of AOs.** Two more types of AOs – one with tails of a short chain of amino acids, a peptide, and the other with a protein-like backbone – 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 to enter the muscles of the heart. To overcome this problem, *Matthew Wood* and his colleagues at the University of Oxford in collaboration with *AVI Bio-Pharma* in Portland/Oregon modified the morpholino AO for skipping exon 23 in mdx mice by attaching short amino-acid chains, peptides, to the antisense structure.

After experimenting with many different combinations of peptides, the researchers developed an optimized *peptide-conjugated morpholino AO* with two 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.

One of the two attached peptides is the *B-peptide*, which helps the AOs to cross cell membranes, and the other is the *muscle specific peptide* MSP, which leads the AO specifically to muscle tissue. The B-peptide consists of 14 amino acids and the MSP of 7. I think you will be interested to see the complete 46 components of the structure of this exon skipping drug for dystrophic mice, written in the abbreviations used by the scientists.

RXRRBRRXRRBRXB – ASSLNIA –  
GGCAAACCUCGGCUUACCUGASAU

Three weekly systemic injections of 6 mg/kg of this opti-

mized 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 report, *Auréli Goyenville* informed me that she and her team in the laboratory of *Kay Davies* in Oxford have used an older version of this type of a peptide-conjugated morpholino AO to treat mdx 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 improved their severe symptoms considerably.

**Exon skipping with peptide nucleic acids.** Another group of AOs, *peptide nucleic acids*, PNAs, 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 PNAs. 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.

*Michael Gait* and his colleagues at the University of Cambridge UK, conjugated, meaning joined, the PNA against exon 23 of mdx mice with a peptide through a thioether bridge containing a sulfur atom. The most effective structure added to the anti-mouse-23 PNA of 25 units was a *PNA-internalization peptide*, Pip2b, with the following abbreviated structure:

RAhxRRAhxRRAhxRIHILFQNdRRMKWHKBAIaC

The intramuscular injection of only 5 micrograms of this PNA-AO into 8-week old mdx mice showed a high number of dystrophin-containing fibers in the single injected mouse muscle. In cooperation with the AVI company, experiments with systemic injections of this very promising exon-skipping drug are being performed (17).

### **Exon skipping with gene transfer.**

**First approach:** The researchers at the Institut de Myologie in Paris, *Luis García* and *Auréli Goyenville* (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 con-

trol sequences, was placed into the DNA of type-2 adeno-associated viruses, AAV. 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 performed 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 U-7 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 *heterogeneous 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 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 because these RNA sequences attract the hnRNPs to the splicing sites, they will induce the skipping of the exon because they interfere with the spliceosomes, sitting at the ends of this particular exon in the pre-mRNA. 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 approval processes possibly required for many or even all AOs used by the normal exon skipping procedures, because the new approach uses only the one “universal” tail structure in addition to the complementary DNA sequence to the sequence of the exon to be skipped.

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.



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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.

A publication on the use of the bifunctional U7snRNA has been accepted by the journal *Molecular Therapy*, its title is “Enhanced exon-skipping induced by U7snRNA carrying a splicing silencer sequence: promising tool for DMD therapy”.

## Personalized therapies and the regulation agencies.

The *Federal Drug Administration*, **FDA**, in the United States, the *European Medicines Agency*, **EMA**, 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 pa-

tients 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 toxicity studies with AOs could shift the reading frame in a normal dystrophin mRNA and, if they are sufficiently effective, give the healthy volunteers Duchenne dystrophy, a dangerous side effect which has nothing to do with “normal” toxicity. 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 toxic effects might appear only in patients but not in people without Duchenne. This makes the standard toxicity 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 hundred million 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.

Duchenne boys have not much time left. Many people therefore think that it is justified to accept the risks that some adverse effects will become apparent only after long-term use. That would mean that the boys whose exon skipping drugs could go through an abbreviated approval process will also have a chance for a therapy before it is too late for them, before most of their muscles are gone.

This summary is based on a new publication by **Eric Hoffman** of the Children’s Medical Center in Washington DC (13).

## Clinical trials

Four clinical trials for skipping exon 51 are described in this most important section of this report. Two of them are *local* ones, in which only one unimportant muscle is treated. This cannot improve the disease of all muscles, they cannot provide a clinical benefit. The two others 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 the hopefully extended lifetime of the Duchenne boys.

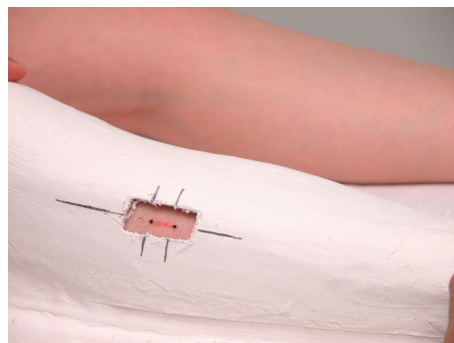
These indispensable steps to the full development of a therapy are just human experiments which also can go wrong. Participation of a sufficient number of boys who need exon-51 skipping is important, but it is not worthwhile for the family to undertake expensive journeys to the trial centers. **Kate Bushby** explains this in her interview starting on page 13.

**Local exon skipping trial in the Netherlands.** The *first in-human trial with the exon skipping technique* was performed under the direction of **Judith van Deutekom, Jan Verschuuren**, and others of the company **Prosensa Therapeutics BV** and the **Leiden University Medical Center** in the Netherlands between January 2006 and March 2007. It was designed to provide a *proof of principle* only and was not expected to result in a therapeutic benefit to the treated boys. It was a *local study* on a small area of a single muscle, the *tibialis anterior* muscle of the shin, which was treated with a 2’O-methyl AO against exon 51 called **PRO051**.

With this type of a chemically modified AO, the Dutch researchers had worked in pre-clinical experiments for

many years and were able to successfully skip dystrophin exons in muscle fibers not only in cell cultures derived from Duchenne patients with different mutations but also in various mouse models, mdx and hDMD, after local and after either subcutaneous or intravenous systemic injections.

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 27<sup>th</sup> of December 2007 in the New England Journal of Medicine with a commentary by Eric Hoffman (18).



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**First systemic clinical trial in the Netherlands.** Between July 2008 and January 2009, Prosensa Therapeutics BV started a *systemic* trial with twelve 5-15-year old Duchenne boys in Göteborg and Leuven with the PRO051 AO against exon 51 injected *subcutaneously*, under the skin.

Experiments with mice had shown that, after subcutaneous injections, the AO spreads throughout the body, and reaches all muscles including those of the heart and lung function. Each boy received one injection at the beginning and then 4 other injections once a week for 4 weeks. This was a dose escalation study with AO doses between 0.5 and 10 mg/kg/injection. The results, which may already show a therapeutic benefit, will be available in the summer of 2009 after full evaluation of all the data. Until the end of March 2009, no serious side effects have appeared.

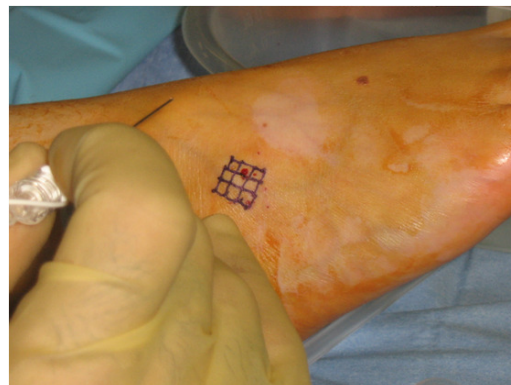
Subcutaneous injections have the advantage that they would not require frequent visits to doctor's offices and hospitals if repeated treatments will later become necessary.

Prosensa Therapeutics BV has acquired gram quantities of the AO against exon 51 in clinical grade quality for the two first trials. Also, AOs with optimized structures to skip exons 43, 44, 45, 46, 50, 52 and 53 have been prepared. The next AO to be developed by Prosensa until marketing will be the one for skipping exon 44 with the first clinical trials to begin in 2009/2010.

Judith van Deutekom said: "We are very pleased with the set up, progress and results of the study so far, especially since no serious side effects were observed to date for each dose group. Our knowledge obtained in this study will be very helpful in the set up of the subsequent phase II/III trial."

**First local clinical trial in the United Kingdom.** Another clinical exon-skipping trial was performed in the UK between the summer 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. 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 being manufactured in clinical grade by the company AVI BioPharma Inc. in Portland, Oregon, and is called AVI-4658 (20).



Seven 12-18-year old Duchenne boys participated. The first two boys received a low dosage of 0.09 mg morpholino AO in 0.9 ml saline, delivered with nine injections of 0.01 ml solution each directly into the *extensor digitorum brevis* muscles at the outside of one foot. The muscle of the other foot received similar injections of salt solution for control tests. The 5 other boys received a 10-times higher dose of 0.90 mg AO in a similar injection schedule. Extensive clinical checks and 2 biopsies were performed before and 30 days after the injections.

In a press release in January 2009, AVI announced that the biopsy data after this first local clinical trial with a morpholino AO showed that new dystrophin was induced

in a dose responsive manner, meaning that with the higher dose, more dystrophin was produced than with the lower dose. The AO drug was well tolerated with no drug-related adverse effects. A manuscript with the full results is ready to be submitted for publication.

**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 (21).

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, 16 still ambulant Duchenne boys in 4 groups of 4 boys, who all need exon-51 skipping, will receive the injections weekly for 12 weeks. The injections are being done sequentially so that a later treatment can be stopped immediately if something happens in an earlier one. All treatments will be finished during the second half of 2009.

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, which will be raised to 4.0 mg/kg for the boys treated at the end of the trial. For a 10-year old boy weighing 30 kg, this would mean that he would receive 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. And it is hoped that a lowest dose can be determined that will induce sufficient exon skipping while being well tolerated by the children without serious side effects.

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 will be taken from the biceps of the boys.

It is expected that the full results of this study can be published at the end of 2009.

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

## Interview with Kate Bushby

### Clinical trials for skipping exon 51 in Duchenne boys.

I recorded this interview in Nicosia on Cyprus on the 21<sup>st</sup> of March 2009 at the 9<sup>th</sup> Congress of the Mediterranean Society of Myology. The following text is an edited and shortened version of the spoken interview. It has been approved by Kate Bushby for you, the patients, your families, and care-givers. My questions are written in italics, Kate Bushby's answers are in normal print.

*Local and systemic clinical trials for skipping exon 51 of the mRNA in Duchenne boys have been and are being performed by the Dutch company Prosensa in Leiden, Liuven, and Göteborg, and by the American company AVI BioPharma in London and Newcastle with the help of the MDEX Consortium of which you are a leading member. Professor Rudolf Korinthenberg at the Children's Hospital in Freiburg told me that he is ready to help either Prosensa or AVI to perform the next large and pivotal clinical trials with his organization of 10 German clinical centers.*

The TREAT-NMD network, of which Rudolf and his team are a key partner, is very pleased about the level of industry interest in the network, in particular for feasibility inquiries, and regarding future studies. It would be fantastic if these future studies were co-ordinated via Rudolf's team.

*If the companies get outside funds from public sources like health departments or so, wouldn't there be problems when they are earning money later?*

There is often collaboration between drug companies and public funders for drugs to be developed. It helps to move the process forward.

*Because I got the merit medal of Germany for my reports from our president Horst Köhler, I can now talk to some German politicians at a high level. I told them that we will need about two million euros for a large trial with 100 Duchenne boys. But it seems to be quite difficult to get anything outside the normal way of applications, and that takes years.*

Yes, getting public money takes a long time, and also the drug companies have to agree that you go down the route of accessing public money for a collaborative project. As you know, exon skipping is mainly being done by Prosensa and AVI. They are developing the technology and they chose to do these ongoing trials. We really hope that partnerships will develop which will allow the development of new trials in the future.

*I saw in a press release of AVI that your local trial has been finished, and that the results are available by now.*

The results have been written up. Our manuscript is in the process of being submitted. The publication should come out soon.

*Your results are positive, aren't they? Are they better than the results the Dutch got in their local trial which were published at the end of 2007 in the New England Journal of Medicine?*

Yes our results are positive. We can't really compare them with the Dutch results, because the way we did the trial was slightly different. For example, we were allowed to take a biopsy from the same EDB muscle of the boy's other leg. One muscle was injected with the antisense drug and the other with only a salt solution, to have a control.

The control is very important, because of the background level of dystrophin in some Duchenne patients. It is necessary to determine that what we are seeing after the treatment is definitely higher than the background. With the low dose, our results were not so good, but with the 10 times higher dose, they were much better. Dystrophin was visible in a significant portion of the muscle fibers.

*Your next trial, the systemic one, has now been started. From what Francesco Muntoni said at ActionDuchenne's meeting in London last November, I calculated that this trial will be ready in about the month of August of this year. Can anything be said already?*

The trial will be finished later than August, but we hope it will be ready this year. Two patients have been injected so far. So it is a bit premature to say anything.

*And will you do multiple biopsies to see whether the drug is active in possibly all muscles?*

No, we are not allowed that. There will be just one biopsy afterwards, from the biceps. This will be compared with a biopsy taken before treatment.

*Do you expect some improvement of the muscle function?*

Not really. But because we have to check that the muscles are not damaged by the treatment we are measuring muscle function and strength. But this is not an efficacy trial. It lasts only for 12 weeks.

*What will happen afterwards, will you need another trial?*

Yes, and that will be the controlled trial, the trial where we have to use a placebo for determining efficacy reliably.

*And this could not be done together with the Dutch to compare the two types of drug at the same time?*

From the academic point of view that would make very great sense. But I don't think the companies would do that. It is most likely that they rather would complete their trials independently.

*But wouldn't one then need to give the placebo to much fewer children?*

Possibly, it would depend on the design of the trial. It is more likely that when both drugs are ready and on the market, the academics could do a comparison.

*The Dutch have just published a paper on the comparison of the two types of the drug where they say that their effect was about the same.*

They were tests, done in the laboratory and with mice but not with humans. And that is not the final answer. It is however very reassuring to see promise with both drugs.

*The Dutch have a so-called first list for the next exons to be skipped. Didn't AVI say in their press release that their next exon whose skipping they will develop will be exon 50?*

It does seem like this is the case. They have told me that they are looking at the deletions of the different patients to determine the next important group of patients whose exons should be skipped.

*Hasn't this already been done by Annemieke Aartsma-Rus in Leiden?*

Our list is slightly different, because it is based on the results of the TREAT-NMD global registry. Around 30 countries are in partnership with TREAT-NMD to contribute to the DMD global database which now has over 8,500 registrants. All of the patients enrolled on these registries

are interested in taking part in trials, and some basic clinical information on them is also available. An overview of the information held will be available on the TREAT-NMD website very soon.

*Will you need new approvals for the next trial?*

For every trial, you need specific approval. From the local to the systemic trial that was quite straightforward. It went faster than for the first trial where we had a new drug and a new technology. The approval for the second, the systemic trial, was much easier for us.

*Do you think that the next steps will also be easier?*

Yes, they will be easier. The regulators would not have approved an early trial if it had not a prospect. So it is important that there are more trials in the pipeline. We will discuss the regulation of the antisense treatment with the EMEA and the FDA agencies next autumn in a meeting led by TREAT-NMD.

*So the talks are going on, and how do they look? Are the regulators interested?*

We are talking to them, but we don't know what will happen. But sure, they are interested in helping us.

*Who are the people who decide to approve or not a clinical trial?*

They are very highly competent pharmacologists and pediatricians. They are experienced people. We know this from the discussions about trials for spinal muscular atrophy.

*Didn't AVI try to do their trials in the United States also?*



**Professor Kate Bushby, MD.**

TREAT-NMD Coordinator  
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I am sure that they have plans to move their trials into the US also. Up until now, the FDA in the States has been

less easy to engage on this issue than the European EMEA.

*Is that the reason you are doing the two trials in the UK?*

Yes, partly.

*In the next trials, can children from outside the UK participate?*

For the current trial, I don't think that it is practical to come from another country, because the children have to be there every week for injections. They have to have lots of blood samples taken. The families have to live near the clinical centers. It would be better to have more trial sites in other countries especially as we move into efficacy studies. Possible trial sites are being identified and screened by TREAT-NMD. We see how complicated it is to take part in a trial with the PTC trial. Families have to put their whole life on hold, even if they live close by. Even for them it is difficult to participate.

*I get requests from all over the world. Even people from Siberia and Argentina would like to come.*

I can imagine that, but they should realize that the trials are only trials. In the 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 always tell them, that the children participating do not get any direct benefit. But if they stay in contact with me, they will get my reports. I have more than 1,000 addresses on my English, German, and Spanish e-mailing lists, so they would find out fast when something is ready for their children.*

I would even say that the people 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..

*Can you say about how long it will take until the first exon-51 skipping drug will be ready? Gerard Platenburg said in my interview with him last July in Philadelphia, it would take about four years.*

Yes it might be something like that. But it is very hard to give even an estimate.

*If one could skip almost all exons, one could treat 83% of all Duchenne boys. But there are other treatment possibilities, like upregulation of utrophin, which then could even be combined with exon skipping.*

Yes, this kind of combination will be one way to enhance efficacy. For the development of potential antisense drugs for the smaller target groups, maybe some private investors or foundations could provide the funds for developing exon skipping drugs for small groups of patients, if the commercial companies cannot do this.

*And the morpholinos are more expensive than the 2'-O-methyls, yes?*

They are both expensive. The company Genzyme sells their drug for the rare neuromuscular Pompe's disease for many thousands of dollars per year. Who knows - exon skipping might be similarly expensive, or it might be much cheaper. If the systemic trials produce spectacular results, then, maybe, some private investors will come along. But when they are not spectacular, this might not happen. Up to now everything is just an experiment for a large group of related new drugs. PTC124 is different, that is just one small molecule the manufacturer has to take care of.

*And it is only for 15% of the patients. Is PTC124 really working?*

Nobody knows yet. We have to wait until the end of the trial.

*Sometimes I am approached by Becker patients. They are already there where the Duchenne boys want to be. But the pharmacological methods might help them.*

Yes, that is true. But they want to improve their situation also.

*Thank you for answering my questions. Would you please conclude this interview with some final words to the Duchenne patients and their families who will read this interview?*

I would just like to say that we have waited for many years to be able to talk about ongoing clinical trials. This is a very exciting new era for this condition.

### 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](http://www.treat-nmd.eu/registry)) and Duchenne Connect ([www.duchenneconnect.org](http://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 already cured, how these boys were diagnosed with what results, how much new dystrophin have they found in the muscles, how much the muscle function has improved, and in which important medical journal the results were published. If that what is offered really had any value, ask yourself why not thousands of families are going there with their sick children. Be careful, otherwise you will lose lots of money and possibly hurt your boy severely.

**Recognition 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 5<sup>th</sup> of February it was given to me by state secretary *Gundolf Fleischer* here in our Black Forest village. I used this opportunity to explain to the about 120 persons present the situation of the families with Duchenne children and to

propose a collaboration between our Dutch and German scientists to perform the next large exon-skipping clinical trial with German patients in Germany. I am now in contact 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, *Ulla Schmidt* and *Annette Schavan*. It will not be easy to get the about 2 million euros for this clinical trial but the correspondence still continues, and at the annual meeting of our Duchenne-parent-project organization *Aktion Benni & Co* on the 16<sup>th</sup> of May at the city of Bochum, the new president of Prosensa, *Hans Schikan*, will discuss details of the proposed Dutch-German clinical trial with us.



And on the 21<sup>st</sup> 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. In about one month, you will be able to see the 55 power-point slides of my presentation on the English section of my internet pages at the address [www.duchenne-information.eu](http://www.duchenne-information.eu). You may download them from there. They are quite detailed and have many animations, so you will understand them without an explanation by me.

**This report has illustrations.** This is my first report with pictures which will help you to better understand my explanations. Quite some time ago, I realized that more women scientists than men are working on finding an exon-skipping therapy for “our” boys. Four of them, *Annemieke*, *Aurélie*, *Judith*, and *Kate* are leading entire teams which again include more women than men. To let you see these four attractive and, obviously, very intelligent ladies, they sent me their photos which I have put into this report.

But there is a fifth photo of a very special lady together with three children. This is *Pat Furlong*, the president of the American Parent Project Muscular Dystrophy, PPM, on a picture taken by me last year at the PPM meeting in Philadelphia. Many of you will know her personally, and most will know what she did and is still doing for acceler-

ating 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!



To conclude these final words, here are my thoughts about the most difficult question I often have to answer:

**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 working place in the Black Forest in Germany.

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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, and Terry Partridge. I am thanking also TREAT-NMD, PPMD, and ActionDuchenne for financial support. Here are their complete addresses and contact information:

**This report will be updated repeatedly.** You can see it – and, after about 6 weeks, translations into German and Spanish – on my internet pages at [www.duchenne-information.eu](http://www.duchenne-information.eu) as well as two interviews and my earlier reports about the two PPMD meetings in Cincinnati 2006 and Philadelphia 2007 and the ActionDuchenne meeting in London 2006. 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.

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### **Our Poster Child.**

This is 5-year old Christian, we found him in 1985, when he was 4 weeks old with our CK screening test for the early detection of Duchenne muscular dystrophy.

He is one of the about half a million Duchenne boys and young men in the world, for whom all our work is done.

## 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.

```

End Exon 49 | Start Exon 50           End Exon 50 | Start Exon 51
---CAG-CCA-GUG-AAG | AGG-AAG-UUA-GAA---AUU-GGA-GCC-U | CU-CCU-ACU-CAG-ACU-
  gln pro val lys | arg lys leu glu   ile gly ala ser|   pro thr gln thr

      hidden stop codon
---GUU-ACU-CUG-UG-ACA-CAA---AAA-CUA-GAA-AUG-CCA-UCU-UCC-UUG-AUG-UUG-GAG---
  val thr leu val thr gln   lys leu glu met pro ser ser leu met leu glu

End Exon 51 | Start Exon 52
---AUG-AUC-AUC-AAG-CAG-AAG | GCA-ACA-AUG-CAG-GAU-UUG---
  met ile ile lys gln lys | ala thr met gln asp leu

```

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.

```

End Exon 49 | Start Exon 51
---CAG-CCA-GUG-AAG | CUC-CUA-CUC-AGA-CUG-UUA-
  gln pro val lys | leu leu leu arg leu leu

      active stop codon
      antisense oligoribonucleotide
      UC-UUU-ACG-GUA-GAA-GGA-ACU
-CUC-UGG-UGA-CAC AAG---AAC-UAG-AAA-UGC-CAU-CUU-CCU-UGA-UGU-UGG--
  leu trp STOP!

End Exon 51 | Start Exon 52
---AU-GAU-CAU-CAA-GCA-GAA-G | GC-AAC-AAU-GCA-GGA-UUU---

```

The exon-skipping antisense oligoribonucleotide, AON 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.

```

End Exon 49 | Start Exon 52
---CAG-CCA-GUG-AAG | GCA-ACA-AUG-CAG-GAU-UUG---
  gln pro val lys | ala thr met gln asp leu

```

No premature stop signal appears in exon 52 or later, but 77 amino acids are missing in the protein, those 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.