

My name is Erwin van Wijk. And I'm affiliated to the Department of Otorhinolaryngology at the Radboud University Medical Center in Nijmegen, as well as to hearing and genes, which is the recently acknowledged Dutch Center of Expertise for Usher Syndrome. And today, I'm going to update you on our most recent findings in the development of genetic therapy for USH2A associated retinal degeneration.

So worldwide approximately 1.7 million people suffer from loss of vision as a consequence of retinitis pigmentosa or in short RP. Of these, more than 400,000 can be explained by mutations in one single gene, which is the USH2A gene. Of these 400,000, approximately 250,000 suffer from blindness only, so non-syndromic retinitis pigmentosa. And approximately 170,000 people suffer from the combination of deafness and blindness, which is named Usher syndrome. And for this condition, currently no treatment is available.

So if we look at it in a clinical way, we see that the USH2A gene is the most frequently mutated gene explaining approximately 50% of all Usher syndrome type 2 patients and in-between 4 to 20% of non-syndromic blindness cases. So Usher syndrome patients type 2 are born with congenital hearing impairments. And approximately around the age of 15 to 20, the first clinical symptoms of loss of vision become apparent. And the first clinical symptoms are night blindness.

At that time, the diagnosis Usher syndrome is being made based on the clinical manifestations. This loss of vision then progresses until the age of approximately 50 to 60 years of age into legal blindness, which leaves in-between for us a window of opportunity at which we can develop some kind of therapy and apply this therapy to the patients in order to prevent or delay the symptoms.

But if we talk about genetic therapy, what is needed for this development? Well, first of all, you would need a strategy. And one strategy could be gene augmentation, so to replace the mutated gene for a healthy copy. However, this provides us with several challenges.

First of all, augmenting a complete new USH2A gene is so far technically difficult. And the reason for this is that the size of the gene, which is huge, is currently too big for the currently used vehicles for delivery of new copies of genes to the retina. Besides that, multiple different USH2A isoforms are present, so different transcripts of the gene. So we don't know yet which transcript will be the most important one, so which one to replace.

An alternative could be gene editing, so repairing a mutation that is found in patients. However, that is currently being developed. Although, the efficiency of the gene editing is still low. And also, off-target effects so repair or interference at a different position in the genome at an unwanted position are being reported. So in general, it's

not yet ready for clinical application and some improvements need to be made.

So ideally, what we would like to do is to interfere on the functional level of USH2A protein or at the transcript level. So if you would do this, we will not alter the balance between the different isoforms that we know of the USH2A protein. And we do not alter the level of general USH2A expression.

Second of all, besides a strategy, we also would need an animal model to test the efficacy of an eventual therapeutic strategy that we have developed. So the most frequently used animal model for this in laboratories is still a mouse model. However, although USH2A mouse models exist in which the gene is being disrupted, these mice show hearing impairment from birth, but remain their vision almost their entire life. So mouse is not the perfect model to model the retinal degeneration in Usher syndrome.

The alternative model that we employ is zebrafish. And why zebrafish? Zebrafish USH2A knockout model shows early onset of retinal degeneration and also early onset impaired visual function. So this really fits more the purpose of our research.

Besides that, all known other Usher syndrome genes that we know in humans are also present in zebrafish. And if we specifically look at the USH2A gene protein structure, those are highly similar between human and zebrafish, which makes zebrafish for our purpose perfect model organism.

So why is a mouse not a suitable model organism and zebrafish is? We think that the explanation for this lies in a very tiny anatomical structure in the light sensitive cells in the retina in photoreceptor cells. And the structure, which is shown with a red circle over here in the schematic overview, and over here these structures in an electron microscopic picture of a human photoreceptor cells are named calyceal processes, which is a difficult name. But we don't know yet what the structure does. What we do know is that all known Usher proteins are present in this particular small and tiny structure in photoreceptor cells and fulfills its function there.

If we look at the same picture of mouse photoreceptor cells, we see that the structures, these calyceal processes, which are present in human photoreceptor cells are absent in a mouse. So the region where Usher proteins fulfill their function are not present in the same light sensitive cells in a mouse retina. In this zebrafish, photoreceptor cells as you can see on the right. These tiny structures are present again, so apparently important for photoreceptor cell function. Therefore, we believe that zebrafish-- that this is the reason that the zebrafish is a suitable model organism to study Usher associated retinal degeneration. Mouse are not.

If we look at the genetic causes of Usher syndrome, a recently discovered important genetic cause resides in a region of the USH2A gene, which is usually not translated into protein. And it resides in so-called pseudoexon 40. And as a consequence, during processing of the gene, an additional piece of sequence is incorporated in the

USH2A gene and is then being translated into a protein. And as a consequence of that, a premature termination of translation into protein occurs resulting in an incorrect and incomplete and nonfunctional USH2A protein resulting in Usher syndrome and USH2A associated retinal degeneration.

So we came up with a strategy to prevent this, well, aberrant incorporation of this additional part of the USH2A gene. And we do this by putting on some kind of-- we call it genetic tape, which masks this part for the machinery that processes the gene into the eventual transcript that is being translated into a functional protein. And as a consequence, this additional part is not being recognized anymore, leaving out this additional part and after translation, a normal, fully functional USH2A protein is being formed. So the problem will be, will be solved.

So the genetic tape, as I just mentioned, are actually antisense oligonucleotides or in short AONs. And these AONs bind to the USH2A gene masking a particular region from being incorporated in the transcript that is eventually being translated into protein. So it prevents the binding of these factors, which are called splice factors and, therefore, results in the skipping or masking of a particular region during a process called splicing.

So the strategy that we have for treating eventually patients with mutation resulting in the incorporation of this additional part, which is named pseudoexon 40 are using AONs to mask this region during the process called splicing. First of all, if we apply this, we would like to study the effect of this particular mutation in patient derived cells, which will be skin derived cells from an USH2A patient. Having this particular mutation and these skin cells are named fibroblasts.

Next, we design AONs, which target this specific region and observe whether these are able to correct for this aberrant incorporation of this part of the gene. Third, we use other patient derived cells. And these are-- which I'll come back to later, will be cells that we can form into photoreceptor-like cells of patients and confirm our AONs, whether or not they can restore aberrant splicing. And finally, we would like to generate a specific zebrafish model that has this particular human mutation and study the effect of this mutation on visual function and whether or not we will be able to also restore visual function by applying these particular and validated AONs.

So first of all, we look in patient derived cells to see whether this mutation indeed has an effect of incorporating this additional part, this pseudoexon 40 or in short PE40. On the left side, the lane named WT, which is wild type. You'll see a DNA fragment. And after analysis, it appears that is the normal USH2A transcript in which exon 40 nicely-- or exon 41 nicely follows exon 40.

In the mutant lane, so this is cells derived from patient having this particular mutation. And now, after sequence analysis, we indeed observed that there's an additional piece of sequence incorporated in the USH2A gene in-between exon 40 and exon 41 and appears to be exactly the known pseudoexon 40 sequence. So indeed, this mutation results in the incorporation of an additional part in the USH2A transcript.

So then we questioned whether or not we would be able to correct for this aberrant incorporation of an additional part of the gene by applying these patient derived cells with this genetic tape, so AONs. For this, we developed two different AONs, which we think could be able to correct this defect. And we named them AON1 and AON2.

On the left side, we see cells obtained from a healthy control individual not having this particular mutation and we do see this lower band appearing. The lane besides that is derived-- is an untreated cell derived from a patient having this particular mutation. So this band is shifted slightly higher up in this plot. And this product marks incorrectly spliced USH2A transcript meaning that this will result in Usher syndrome.

So by applying these particular cells with AON1, we do see that we approximately can restore this higher product into the lower product by 50%, meaning that in 50% of the transcript, we could provide incorporation of this additional piece, so restoring the original fragment. By applying AON2 in the next lane, we also do see this effect only it is less efficient. So approximately 80% of transcripts still contain this aberrant part and 20% of transcripts lack this additional pseudoexon 40.

Then we have a control genetic tape, which does not recognize USH2A at all. That's how it's designed. And indeed, when we apply this SON as we name it, we don't see an effect at all, which is expected. And if we then combine both AON1 and AON2 together, which is the final lane, we do see that in approximately 95% of all transcripts we can indeed correct this aberrant incorporation of this additional part meaning that this treatment, when we combine both AON1 and 2, is highly efficient. So it's very worthwhile looking into a little bit further.

So if we talk about eventual delivery, still a lot of things need to be done, toxicity study, et cetera. But eventually, we will need to deliver it to the organ of interest, so the eye. So how are we going to deliver this genetic tape?

We have two options. First of all, we can deliver it in a naked way. This will mean that we will probably have to perform intraocular injections on a repetitive basis. And we anticipate so far based on the knowledge that we have from literature, that we will need to repeat this treatment approximately three to four times a year.

An alternative way could be to package these genetic tapes into a virus, which could be an Adeno-associated virus or a lentivirus. These are vehicles that are currently also being used for gene augmentation therapy, so gene replacement therapies. Then this will be a subretinal injection, so an injection behind the retina. And presumably, this will be a single injection. Which modes and route of delivery will be most efficient and also the safest? To determine this, we will need to perform, obviously, more future research.

We are now at the stage of translating these findings into the proper context. So you can imagine that we are unable to take out retinal cells from an USH2A patient. These cells are-- well, we cannot reach them. So there's

one technology and that is stem cell or iPS technology, which is recently being improved. And using this technology, we can redirect skin derived fibroblasts from an USH2A patient via stem cell technology into photoreceptor-like cells, which then contain the perfect genetic environment of our patient to study.

And these cells in the cellular model is the model that most closely resembles the human retinal situation. So these are very worthwhile studying. If we have generated photoreceptor-like cells derived from the patient, so containing this mutation, we will apply these genetic tapes. And then study whether or not these AONs are indeed functional, so can be correct for splicing also in this specific cell types. And in addition, we are currently generating zebrafish model, which contains this particular human sequence and study the effect of the mutation in the zebrafish retina and if we can also correct for this mutation there.

Further on, we will study in these cells the mode of delivery as mentioned compare naked delivery via a viral based delivery and see which one is most efficient and most safe. We will also evaluate whether or not this treatment is specific or if we can also detect some off target effects. And we will determine whether or not this treatment is safe or that we maybe observe some toxicity effects.

In case we have completed all this, this will form the basis for the initiation of phase 1, 2 clinical trials, which we anticipate to commence hopefully in 2018 or otherwise 2019, which is an ambitious goal. However, in this field, you need to be ambitious. That's at least our philosophy.

So in summary, we have USH2A patients having a deep intronic mutation in USH2A resulting in the incorporation of an additional piece of gene that is being translated in USH2A protein resulting in aberrant nonfunctional protein, and as a consequence in Usher syndrome. We have developed genetic tapes or antisense oligonucleotides by which we can interfere with this splicing machinery. And we can correct for this aberrant incorporation of part of the gene.

We have modeled this in a minigene splice assay and shown that this works and have confirmed this in patient derived material, so fibroblasts. And now, for evaluating the big preclinical efficacy and safety, we are heading towards photoreceptor-like cells that we generate from patient derived skin fibroblasts and also zebrafish models. And this is exactly the stage at which we currently are, which forms the basis for eventual phase 1 and 2 clinical trials. And hopefully, in the future, we can go back to the patients, in order to prevent or delay of retinal degeneration in USH2A patients having this particular mutation.

Now, I'll end my presentation by acknowledging the people that did all the work. First of all, my team. Second of all, our collaborators. And last, but not least, all the patient organizations and foundations that made this research possible.