194th ENMC international workshop.
3rd ENMC workshop on exon skipping:
Towards clinical application of antisense-mediated exon skipping
for Duchenne muscular dystrophy
8–10 December 2012, Naarden, The Netherlands

Annemieke Aartsma-Rus \textsuperscript{a,⁎}, Francesco Muntoni \textsuperscript{b}

\textsuperscript{a}Department of Human Genetics, Leiden University Medical Center, Albinusdreef 2, 2333 ZA Leiden, The Netherlands

\textsuperscript{b}Dubowitz Neuromuscular Centre, Institute of Child Health & Great Ormond Street Hospital, 30 Guilford Street, London WC1N 1EH, UK

Received 29 May 2013; accepted 17 June 2013

1. Introduction

Twenty-seven participants from 9 countries (Australia; Belgium; England; France; Germany; Italy; Japan; The Netherlands; USA) attended the third ENMC workshop on exon skipping “Towards clinical application of antisense-mediated exon skipping for Duchenne muscular dystrophy.” The topic of this workshop was on ‘Streamlining the development path of exon skipping compounds’ and followed the formula of similar workshops held in 2004 on ‘Antisense oligonucleotides in DMD’, which focused on intramuscular administration of antisense oligonucleotides and in 2007 on ‘Planning Phase I/II Clinical Trials using systemically delivered Antisense Oligonucleotides in Duchenne Muscular Dystrophy (DMD)’.

The workshop was organized with the support of Parent Project Muscular Dystrophy (PPMD), Duchenne Parent Project (the Netherlands) and Parent Project Onlus (Italy), in addition to the ENMC, and was attended by representatives of the three companies involved in the clinical development of exon skipping, Prosensa and GlaxoSmithKline for the 2′-O-methyl phosphorothioate RNA modified (2OMePS) AONs and Sarepta (previously AVI Biopharma) for the phosphorodiamidate morpholino oligomers (PMOs), as well as patient representatives, academics (scientists and clinicians) and an expert associated to the European Medicine Agency (EMA) who, while participating as an individual brought broad regulatory perspective to the workshop.

1.1. Aim of the workshop

Duchenne muscular dystrophy (DMD) is a severe, progressive muscle-wasting disorder that affects ~1 in 5000 newborn males \cite{1,2}. The disease is caused by mutations in the dystrophin encoding \textit{DMD} gene. The dystrophin protein normally provides muscle fibers with stability during contraction by linking the cytoskeleton to the extracellular matrix. In DMD patients mutations disrupt the open reading frame, leading to a prematurely truncated dystrophin protein that cannot fulfill its linker function. By contrast, the less severe Becker muscular dystrophy (BMD) is caused by mutations that maintain the reading frame, allowing the production of internally deleted proteins that have (partially) maintained their linker function \cite{3,4}. Corticosteroids are the only drugs that have shown a beneficial effect, however treatment is only symptomatic \cite{5,6} acting to slow disease progression. Despite improved care, most DMD patients die by the third or fourth decade of life due to respiratory or cardiac complications, and in the latter stage of their condition are highly affected by severe and generalized muscle weakness that precludes almost all voluntary movement.

In the absence of curative treatment, the exon skipping approach aims to convert the severe DMD into a milder BMD phenotype by modulating the pre-mRNA splicing of the dystrophin transcript. This can be achieved with antisense oligonucleotides (AONs), pieces of chemically...
modified DNA or RNA that target specific exons, interfere with the splicing machinery and cause them to be excluded (skipped) from the mature mRNA. In this manner the open reading frame can be restored, allowing the production of a partially functional BMD-like dystrophin rather than a non-functional DMD-like dystrophin [7]. After extensive optimization in patient-derived cell cultures and multiple mouse and dog models (reviewed in [7]), proof of concept (dystrophin restoration) has been achieved after local intramuscular injections and systemic treatment for both 2OMePS and PMO AONs [8–11]. Currently, AON-induced exon skipping is being tested in hundreds of DMD patients in late phase clinical trials and is viewed by many as the most promising approach for DMD.

1.2. The issue of mutation specificity

Annemiek Aartsma-Rus introduced the issue of applicability and mutation specificity. Exon skipping aims to restore the reading frame. Therefore, different exons have to be skipped for patients with different mutations. Exon skipping will not apply to all mutations, as a dystrophin protein lacking the dystroglycan binding domain (encoded by exon 64–70), all its actin binding domains (encoded by exon 2–8 and 35–44) or over 75% of its central rod domain is not functional. Fortunately, DMD mutations (mostly deletions) cluster in a hotspot region (between exon 42 and 55), and these are generally amenable to exon skipping. Due to the clustering of mutations, skipping certain exons applies to larger groups of patients, e.g. exon 51 skipping would apply to the largest group of patients (13%) and is currently being tested in clinical trials. However, the majority of patients theoretically eligible for exon skipping requires the skipping of an exon that would only benefit a very small subset of patients (less than 0.5% of all patients) [12]. As each AON may be considered as a separate drug, the clinical development of AONs targeting these exons will be relatively lengthy and expensive, but especially challenging when the issue of demonstrating clinical efficacy for very small population of affected individuals is considered. The aim of the ENMC workshop was to discuss strategies to allow a streamlined way forward involving all key stakeholders (patient representatives, academic researchers, clinicians, industry representatives, with the insight of the regulatory authorities).

2. State of the art

2.1. Currently ongoing clinical trials for exon 51 skipping

Multiple clinical trials are currently ongoing for drisapersen (a subcutaneously delivered 2OMePS AON targeting exon 51, previously known as AVI4658, developed by Sarepta, previously AVI Biopharma).

Padraig Wright (GSK) indicated that drisapersen development is being done thoroughly, since it is potentially the first AON drug intended for lifelong treatment of a childhood onset disorder. Furthermore, due to the mutation specificity of the approach, it is also potentially the first of a family of AON drugs for DMD patients. It has been postulated previously that after the first two AON compounds are developed fully, a faster development path for AONs of the same chemistry could be considered [13]. Multiple drisapersen trials are currently ongoing (see Table 1). Furthermore, GSK is planning additional trials for drisapersen in non-ambulant boys and adults and in very young children.

For the open label extension study following the escalating dose study [9] all 12 patients have now been treated for over 3 years, providing a wealth of information on long term safety and tolerability. Patients were treated with weekly subcutaneous injections for the first 72 weeks, after which they were off treatment for 8 weeks, followed by an intermittent dosing scheme of 8 weekly injections and a treatment free period of 4 weeks. Injection site reactions were the most frequently observed side effect. This is a common observation for 2′-O-modified PS AONs that are delivered to human subjects though the subcutaneous route. Proteinuria was observed in all patients, but at levels that do not cause clinical concern (renal function is unaffected); indeed the proteinuria is reversible during off treatment periods and although it reappears following treatment re-initiation, its severity does not appear to increase on the longer term. Transient thrombocytopenia has been observed in the open label extension study following the published dose escalation study [9], but no levels reached $<75 \times 10^9/L$.

The patients involved in this trial have been followed with the 6 min walk test. Between the end of the dose escalation trial and the onset of the OLE trial, 2 boys had lost ambulation. Of the remaining 10, 2 have lost ambulation during the OLE trial, while the remaining 8 have remained ambulant; in 7 of these boys the distance walked in 6 min has been maintained or improved for over 3 years compared to the baseline. Despite the fact that a placebo group was not present in this study, the data are encouraging as they diverge significantly from the natural history studies.

Recruitment for the other studies has been completed and results of the phase III study are expected in Q4 of 2013. The results of the dosing regimen study have recently been presented at a meeting (April 11, Cold Spring Harbor, NY, USA). In exploratory study, the continuous treatment arm (6 mg/kg/weeks, n = 18) showed a statistically significant difference from placebo (n = 18) on 6MWD at 24 weeks (mean, 35.09 m; 95% CI, 7.59–62.60 m), p = 0.014), with trends supportive of efficacy in other timed function tests and the North Star Ambulatory Assessment (NSAA). Also at 48 weeks an
encouraging trend differentiating from placebo (35.84 m \([-0.11 \text{ to } 71.78 \text{ m}], p = 0.051\)) was observed. The intermittent treatment arm \((n = 17)\) did not separate from placebo at week 24, though by week 48 there was a clinically meaningful trend from placebo on 6MWD (27.08 m \([-9.83 \text{ to } 63.99 \text{ m}], p = 0.147\)), supported by trends in timed function tests and the NSAA. There was little change in muscle strength at either time point for either treatment arm. Drisapersen was generally well tolerated, with the majority of adverse events related to injection site reactions and proteinuria (John Kraus, personal communication).

The dose comparison study is ongoing in the USA. Results from this study are anticipated in Q1 2014. More than 100 patients have been enrolled in the second open label extension study. Notably, out of the over 300 patients currently involved in drisapersen trials the retention rate has been very high (96%).

Jerry Mendell presented on ongoing eteplirsen trials (coordinated by Sarepta). Following the dose escalation study which tested doses of up to 20 mg/kg eteplirsen, [8] the current trial compares 30 and 50 mg/kg doses. For the first 24 weeks groups of 4 patients received 30 or 50 mg/kg eteplirsen or placebo. Then, the placebo group was stopped and split into two groups of 2 patients receiving either 30 or 50 mg/kg. Biopsies were obtained before treatment, after 12 weeks for the 50 mg/kg group and 2 placebo treated patients, after 24 weeks for the 30 mg/kg group and 2 placebo treated patients and after 48 weeks for all patients. Patients were unblinded to the arm in which they participated in week 36.

Dystrophin analysis was performed by immunofluorescent analysis and the percentage of dystrophin positive cells was assessed for each patient based on at least 1000 muscle fibers by blinded investigators. In the patients treated for 48 weeks, the percentage of dystrophin positive fibers had increased significantly to 52% and 42% for the 30 and 50 mg/kg groups, respectively. For the patients receiving first placebo and then treatment for 24 weeks, the percentage increased to 34% and 43% for 30 and 50 mg/kg groups. No dystrophin was observed for the 50 mg/kg group after 12 weeks of treatment, while a significant increase was observed for the 30 mg/kg group after 24 weeks of treatment. These data, which will need to be supported by detailed analysis of the level of dystrophin produced by the fibers, suggest that chronic administration of a lower (30 mg/kg) dose is more efficient than a higher (50 mg/kg) dose administered over a shorter period of time, echoing data from preclinical models [14].

These patients were followed with the 6 min walk test as well. For patients receiving 50 mg/kg the distance they were able to walk stabilized, while it decreased for the group receiving placebo (24 weeks) and decreased further for the first 12 weeks of treatment (until week 36) and then stabilized. For the 30 mg/kg group 2 patients (identical twin brothers) quickly lost ambulation in the early phase of the study and were removed from further analysis. Aside from a single proteinuria event no drug related adverse events were reported.

2.2. What is known for 2OMePS and PMOs from preclinical and clinical trials

In preclinical and clinical pharmacokinetic/pharmacodynamic studies AON plasma and tissue levels are monitored and related to molecular and clinical outcome measures. Sjef de Kimpe presented the various 2OMePS AON detection systems used at Prosensa to assess plasma and tissue levels, whole body distribution, tissue localization, and metabolites, such as an hybridization ELISA-like assay, radioactive screening, in situ hybridization, and HPLC (with mass spectrometry). Similar systems are also in place (HPLC) or being developed (ELISA-like system) to detect PMOs. Each system has advantages and limitations. Notably, these systems cannot reveal in which compartment of the tissue the AON is located (e.g. in the muscle fibers or in the interstitial space or fibro-adipose tissue or cytoplasmic versus nuclear location).

Pete Sazani presented pharmacokinetic studies with different PMOs. Studies in animal models show very good tolerability even at very high doses of PMOs [15] and experience so far suggest there does not seem to be dose limitations due to safety issues in humans either. As PMOs are non-charged and small enough to be filtered out by kidney they have a very short serum half-life (2–3 h) and the vast majority is excreted via urine on the
first pass (~95% of PMO is present in urine within 30 min). Only minimal amounts are taken up by muscle.

Studies in primates using intravenous or subcutaneous doses of 5–320 mg/kg/kg for 12 weeks revealed good tolerability of PMOs. On histology, some basophilic material (probably the PMO) could be observed in kidney tubular epithelium, but this did not affect kidney function and disappeared with turnover of kidney epithelial cells. When comparing data in rodents, non-human primates and humans it appears that the dosing needs to be scaled by weight (i.e. the same dose in mg/kg is used for different models regardless of their size and there is no allometric conversion as is the case for 2OMePS AONs).

Ed Kaye presented data on PMOs tested in healthy volunteers and patients suffering from other diseases such as cancer and hepatitis. These PMOs were 25–30mers and the PK properties were similar for different PMOs regardless of size and sequence composition. Furthermore, PK was similar for different species, as also already observed for DMD PMOs. For each PMO higher doses resulted in higher half-lives, although the doses tested were relatively low (up to 90 mg in total). Excretion takes place primarily through urine for all PMOs tested so far.

In addition to regular PMOs some preclinical development has been done for peptide linked PMOs (p-PMOs). Most advanced studies have been performed with the B-peptide and the PMO-plus compound, which unfortunately both resulted in significant kidney toxicity in non-human primates. While the development of these p-PMOs has been discontinued, both Sarepta and the MDEX consortium in UK are pursuing the search of other p-PMOs with an acceptable therapeutic index.

Sjef de Kimpe presented PK and absorption, distribution, metabolism and excretion (ADME) data on 2OMePS AONs. Also here profiles were similar for different AONs. However, unlike the PMO, this chemistry is charged and binds serum proteins with low affinity, which prolongs their circulation time, half-lives, and uptake by muscle. The length of the AON typically influences the strength of serum protein binding.

Biodistribution studies revealed that except for the brain and the spinal cord, 2OMePS AONs are taken up by all tissues upon systemic delivery. They could still be detected 28 days after delivery, albeit at lower levels. The half-life appears to be 4 weeks for most tissues, while clearance is quicker from kidney. PK analysis in patients revealed that 2OMePS AONs are quickly cleared from the plasma and then distributed to muscle and other tissues. Multiple dosing leads to increasing pre-dose trough levels. The serum half-life in the second phase (after uptake by tissues) in patients is also ~4 weeks.

Metabolite analysis with HPLC for 2OMePS AONs revealed that after 24 h mainly the parent AON was present, while after 7 days some metabolites were evident (missing e.g. n–1, n–2, n–3 etc.). The behavior of the metabolites is very predictable: the n–1 and n–2 AONs still induce some exon skipping, while those with more nucleotides missing are inactive.

Data were presented on 2OMePS AONs to induce DMD exon 45 and 53 skipping. The safety analysis gave very similar results for both AONs and when compared to PRO051. In monkeys pro-inflammatory class effects are typically observed at high doses of 2OMePS AONs, e.g. complement activation, lymphoid proliferation, release of cytokines and chemokines and the detection of split complement factors. However, this appears to be a species specific effect. In humans mainly injection site reactions are observed, while a rise in systemic inflammatory response factors or a decrease in complement are not characteristic findings. 2OMePS AONs accumulate in kidney (tubular epithelium) and liver. Proteinuria is often observed in 2OMePS AON treated humans, but liver and kidney function parameters are generally normal.

Sjef de Kimpe also presented on preclinical safety studies for 2OMePS AONs targeting exon 51 and exon 44 (up to 9 months data available) and exon 45 and exon 53 (3 month data available). These did not reveal novel toxicity and confirmed that each 2OMePS AON behaved in a similar fashion. When discussing the possibility of an expedited development path for additional 2OMePS AONs, the concern of regulators is toxicity outside the well documented class effects. However, for DMD the risk for this is minimal as (1) exaggerated pharmacology (the drug working too well) is not an issue, since the effect of the AONs is limited by the number of dystrophin transcripts (2) off target effects have not been observed and remain unlikely since the AONs are designed with high DMD gene sequence-specificity and 2OMePS chemistry is sensitive to mismatches (3) the structural diversity of 2OMePS AONs is limited compared to small molecules (4) the 2OMePS compound closely resembles natural RNA nucleotides (5) the AONs have predictable metabolites with similar structural constraints and (6) AON metabolites have either reduced biological activity (n–1, n–2) or no biological activity (when more nucleotides are removed), so derivatives are not expected to be a safety concern. Thus for future 2OMePS compounds 3 month toxicity studies would give adequate information for risk assessment. This developmental plan has been considered acceptable.

Art Levin presented the comments of the FDA on the NDA/safety data on the mipomersen study done by ISIS Pharmaceuticals and future implications for AON therapeutics. Mipomersen is a 20mer 2′-O-methoxymethyl phosphorothioate/phosphorothioate gapmer AON that works through RNase H cleavage of ApoB transcripts of patients with familial hypercholesterolemia (a rare disease leading to death in the 3rd decade of life due to cardiovascular complications). The FDA advisory panel has recommended approval and this has resulted in
approval in the USA, while approval in EU has not been granted.

The data from the clinical trial revealed that the compound is active: LDL levels were lower after 5 weeks of treatment. Some side effects were observed: proteinuria (as anticipated for this class of compounds) and increased ALT levels and in liver fat (as a consequence of ApoB inhibition). Other reported side effects were injection site reactions and flu-like symptoms, which resulted in discontinuation of 10% of patients involved in the trial. No activation of complement or increases in complement split products were detected in any of the patients, while this was observed for monkeys in preclinical safety tests (at 7 times the clinical exposure tested in patients). Malignant neoplasms were observed in some treated patients, but the incidence was similar to that observed in analogous patient populations. In many patients antibodies to mipomersen (or antibodies able to bind mipomersen) were observed, especially in patients with high plasma trough concentrations. There was no apparent effect of these antibodies on safety or efficacy.

3. Response to treatment

Francesco Muntoni presented the published eteplirsen dose escalation trial that took place in the UK in which 6 groups of children received ascending doses of the PMO to induce exon 51 skipping. In the 2 higher doses (10 and 20 mg/kg for 12 weeks) 80% of patients appeared to show a response in terms of protein and skipping although there was variability, with some good responders and some poor responders [8]. Various causes for the difference in response after this 12 weeks trial were assessed. Plasma levels per se or genotype did not appear to be a major contributory factor. To assess whether non-responsiveness was caused by intrinsic differences between patients, an in vitro test on patient-derived cell cultures was performed. Fibroblasts were isolated from a skin biopsy and forced into myogenesis by MyoD transduction and then treated with AONs and studied using a quantitative PCR test. No difference in exon skipping levels was observed between responsive and non-responsive patients. This further underlines that all patients had the potential to respond and that their non-responsiveness was probably due to a lack of PMO in the muscle that was biopsied. Indeed differences in the amount of dystrophin positive fibers in different fascicles of the same muscle were noted in this study, and have been previously well documented in the mdx mice, indicating the limitation of considering patients as “responders or non-responders” based on a single and limited muscle biopsy sample. Indeed it has been reported in the mdx mouse model that multiple treatments over time with a low PMO dose results in more dystrophin positive cells than a single high dose [16]. This indirectly suggests that a number of non-responders in this trial might have responded if they had been treated for longer. However, it is clear that response to treatment is a multifactorial issue, of which drug exposure is most likely one component, but for which many components are still unknown.

3.1. Anticipated response

The exon skipping approach aims to convert the DMD into a BMD phenotype. Thus, more information on the natural history of BMD patients is warranted to allow better prediction of the possible beneficial effects of exon skipping.

Jan Verschuuren presented a comprehensive study in a cohort of 27 BMD patients, from whom a biopsy was taken at the time of muscle function assessment. Dystrophin levels were assessed with quantitative western blotting and varied between 17% and 74% of normal. No correlation was found between dystrophin levels and muscle strength, the mean fat fraction (assessed by MRI), or disease progression (as defined by the age of disease milestones). A significant subset of patients carried an exon 45–47 deletion (13 patients). Also for these patients, who expressed varying levels of an identical internally deleted dystrophin, no correlation was observed between dystrophin levels and muscle function, quality or strength. However, for this subgroup a significant correlation between age and muscle strength and between age and mean fat fraction was observed. This suggests that the disease progresses at a relatively homogeneous way in patients with a similar mutation, but that disease progression is not directly correlated with dystrophin levels, provided these levels exceed a minimal threshold.

Francesco Muntoni presented work on quantification of dystrophin levels assessed with immunofluorescent staining and western blot for DMD patients needing exon 51 skipping and their BMD counterparts. For these patients, a good correlation between dystrophin levels and severity was found [17]. However, for BMD mutations as those that would result from exon 44 skipping and DMD mutations requiring exon 44 skipping, the correlation was less obvious, confirming the data presented by Jan Verschuuren on this latter population of patients. Moreover many DMD patients with a deletion flanking exon 44 or exon 45 have higher levels of trace dystrophin expression, presumably due to spontaneous exon 44 or exon 45 skipping, compared to DMD with deletions correctable by exon 51 or 53 skipping. The trace dystrophin levels in some of these individuals is apparently sufficient to ensure a slower decline of muscle power and Francesco Muntoni highlighted that several of these patients followed an intermediate or even BMD course, instead of the expected DMD. Judith van Deutekom emphasized in her presentation that it is currently not yet known how much dystrophin is required for a beneficial effect. It seems that below a certain threshold there is linearity between pathology and dystrophin levels. Mouse models do not need a lot of...
dystrophin for improvement of the phenotype [18] or survival [19]. However, upon stress such as eccentric contractions, higher levels of dystrophin are needed to prevent muscle degradation [18]. One should not forget that there is a difference between BMD patients who have life-long production of partially functional dystrophin and DMD patients treated with AONs, who started production of BMD-like dystrophins later in life. Thus, AON treated DMD patients may not be exact phenocopies of BMD patients. To better predict the potential effect of AON treatment on DMD patients, we can learn from the ongoing trials with eteplirsen and drisapersen. These induce exon 51 skipping and thus primarily address patients with deletions in the central rod region – for which it is known that in-frame mutations are associated mostly with a mild BMD phenotype [20]. Hopefully the data obtained from exon 51 skipping studies can later be extrapolated to support smaller, underpowered studies for additional exons.

4. Outcome measures

Especially for future trials that of necessity will have to be conducted in smaller groups of patients, good outcome measures will be crucial.

4.1. Dystrophin measurements

It is known that DMD patients may have variable trace amounts of dystrophin present in their muscles [21]. The levels of this trace dystrophin may differ per patient, and complicate the quantitative analysis of early dystrophin restoration in muscle biopsies after treatment. When assessing the percentage of dystrophin positive fibers, generally one subtracts the percentage of dystrophin-positive fibers observed in the pre-treatment biopsy from those of post-treatment biopsy. When assessing the relative dystrophin levels per fiber in a fiber population one can use measures based on intensity of the fluorescent signal [22]. Afrodite Loubarkos presented the approach currently used at Prosensa to assess dystrophin restoration in muscle biopsies. She stressed the importance of standardizing handling and shipping of biopsies, e.g. through training people locally obtaining and handling the biopsy, using experienced couriers, sending duplicate biopsies at two different time points and also having trained personnel receive and process the biopsy.

H&E staining and spectrin staining of cross sections is done to ensure good quality biopsies. Dystrophin restoration at the membrane was monitored using immunofluorescence and computational automated image analysis that measures the varying dystrophin intensity per individual fiber, and objectively produces a histogram of the dystrophin distribution in the fiber population. On average 1000 fibers from 2 sections are analyzed. In DMD patients treated with PRO044 comparisons of the dystrophin intensity distribution histograms of pre- and post-treatment muscle biopsies showed increases in dystrophin intensity of entire muscle fiber populations post-treatment.

4.2. Functional outcome measures

The exon skipping approach aims to convert a severe disease into a milder disease. Therefore, the patients will not be cured. Rather the disease progression will be slowed down and preferably stopped. Significantly slowing down disease progression could be sufficient for approval by regulators. However, obtaining this evidence is challenging. The 6 min walk test is currently the only validated test used to assess muscle function in neuromuscular disorder patients (like DMD). When this test is performed in DMD patients generally the distance they walk in 6 min increases until 7 years, after which it plateaus and then decreases as disease progresses. However, there is a lot of variation between patients in the level at which they plateau and the time at which they start decreasing.

Nathalie Goemans presented on efforts in her Institute with the 6 min walk test in 90 healthy boys ages 5–12 years [23]. The distance walked in 6 min (6MWD) increased with age. Test-retest reliability was good, even in the younger ones. Then sixty-six steroid treated DMD patients of varying age groups were studied in a natural history study [24]. Muscle function tests were performed every 6 months. Large differences in 6 min walk test results were observed between individual boys. On average the distance walked in 6 min increased until boys were 7.5 years old, then the distance plateaued and eventually declined precipitously, but the standard deviation increased, as timing and rate of decline was different for different DMD patients. On average a decline in 6MWD of −43 m, −64 m and −125 m at, respectively 1, 1.5 and 2 years follow-up was observed. This decline was steeper in boys above the age of 7.5 years, however numbers in the <7.5 year groups were consistently lower.

The decline observed in the 6 min walk tests was also seen for other timed tests, although the onset and rate of decline differed for different tests (e.g. time to rise, 10 meter walk test etc.). Unfortunately, no validated scale is available yet to assess upper limb function. Eugenio Mercuri is currently leading a working group on the interpretation of the clinical outcome in trials involving a small number of patients needs to be interpreted with particular caution, also paying attention to the size of the control group.

The decline observed in the 6 min walk tests was also seen for other timed tests, although the onset and rate of decline differed for different tests (e.g. time to rise, 10 meter walk test etc.). Unfortunately, no validated scale is available yet to assess upper limb function. Eugenio Mercuri is currently leading a working group on the interpretation of the clinical outcome in trials involving a small number of patients needs to be interpreted with particular caution, also paying attention to the size of the control group.

The decline observed in the 6 min walk tests was also seen for other timed tests, although the onset and rate of decline differed for different tests (e.g. time to rise, 10 meter walk test etc.). Unfortunately, no validated scale is available yet to assess upper limb function. Eugenio Mercuri is currently leading a working group on
assessment and validation of upper limb function tests in muscle disease patients.

Jerry Mendell presented data on the 6 min walk tests and showed that results from these tests correlate very well. However, the 2 min walk test results appear generally to be more reliable, probably because there is less dropout of smaller boys (due to lack of concentration) and older boys (due to fatigue).

Jerry Mendell also presented the correlation between muscle strength and function. For DMD patients a significant correlation was observed between quadriceps strength and performance in the 6 min walk tests, North Star analysis and the 10 m walk test. Thus muscle strength appears to predict muscle function. However, no significant correlation was found between strength and stair climbing and the correlation between strength and function became less when disease progressed.

Although the current position of the European regulators is still that improvement in both function and strength, captured by validated outcome measures, will be needed for approval, improvement only in muscle function could be considered, based on the robustness of the results and provided no deterioration in muscle strength is seen.

### 4.3. Surrogate markers and biomarkers

Currently the most important molecular outcome marker used to confirm treatment effect is dystrophin restoration in a muscle biopsy. It is clear that doing multiple muscle biopsies over time or from different locations or muscles is too invasive and untenable for patients with a progressive muscle-wasting disease. Based on the facts that dystrophin expression can be patchy, responses varied for different muscle groups in systemic preclinical animal studies. In addition, the correlation between levels of different internally deleted BMD dystrophins and muscle function or strength is not yet fully understood, dystrophin is therefore currently considered a pharmacokinetic biomarker rather than a surrogate marker.

To support the detection of a therapeutic effect, additional, and preferably less invasive biomarkers need to be identified and validated. These could be serum or plasma proteins, the levels of which correlate with muscle function or non-invasive methods to assess muscle quality such as magnetic resonance imaging. Based on guidelines from EMA these supportive biomarkers for DMD should correlate with muscle function and ideally also strength.

Terry Partridge presented data on mass spectrometry to identify serum biomarkers. Several peptides, proteins and lipids were identified that were present at different levels in DMD compared to control sera. However, further investigation in larger cohorts will be needed to validate these findings.

Annemieke Aartsma-Rus presented data on serum matrix metalloproteinase 9 (MMP-9) as a potential biomarker for DMD. MMP-9 levels have already been reported to be elevated in DMD patients and the mdx mouse model and to increase over time [25]. Levels are also increased in BMD patients. Notably, there is huge variation of serum MMP-9 levels between patients, but for individual DMD patients levels increase when patients become older. This is in contrast to e.g. creatine kinase levels, where levels are increased in early stages of the disease but go down later when patients lose muscle mass. Thus for creatine kinase levels lower serum levels upon treatment can either mean the muscle quality has...
Magnetic resonance imaging (MRI) can be used as a non-invasive method to assess muscle quality. Jan Verschuuren presented additional advantages of MRI over a muscle biopsy to assess muscle quality. A biopsy provides only local information for a single muscle, while MRI provides more global information for multiple muscles. Using different MRI analyses, different parameters can be assessed, such as morphology and hypertrophy using T1 weighted images, fatty infiltration using Dixon, edema using T2 weighted images, fiber architecture using diffusion tensor imaging (DTI) and tissue metabolism using MR spectroscopy [26]. The limitation of using MRI as an outcome measure for trials is that often trials are short (weeks), while the changes in muscle quality likely take longer (months). Another disadvantage is that generally an MRI takes a relatively long time (~1 h), which is burdensome for younger DMD patients (especially since attention deficit hyperactivity disorder is more prevalent in these patients [27]). Nevertheless, for longer term trials and post marketing studies MRI will most likely prove an invaluable tool to assess the effect of treatment on muscle quality over time.

5. Towards developing exon skipping for small subsets of patients

At the moment each AON is observed by the EMA as a different entity, and thus has to be tested separately. For exons or combinations of exons that would only benefit small numbers of patients, however, clinical development will be very challenging. With current regulations it will not be feasible to test AONs targeting different exons in a single trial (e.g. in a group of patients with small mutations in in-frame exons). However, once multiple AONs of a certain class chemistry have been shown safe and effective, conditional approval of additional AONs of the same chemistry may become possible.

One of the main questions is whether placebo groups will be needed for these small subsets of patients. As discussed in Section 4.2, performing tests to assess functional outcome in small patients groups has the potential to lead to erroneous results. It is possible to compare the results to historical data of natural history studies, but this is generally not very reliable, due to care standards that improve over time. Thus ideally a common dataset of placebo-treated patients should be used (e.g. using data obtained in exon skipping trials for which a large placebo group was included). This would provide the regulators with a control group that does not differ much from the patients involved in smaller trial. Of course this would require collaboration between different industry partners, who would have to make sure that clinical protocols are comparable and who would have to be willing make the placebo dataset available to each other. In addition there are several longitudinal natural history studies currently ongoing in the Netherlands, Belgium, UK and Italy. Acquiring data from these natural history studies is a collective collaborative effort – although not as powerful as a placebo group – and should be encouraged as well, because in due course there might be no placebo treated boys recruited into clinical trials.

6. Developing improved chemistries and delivery methods

Additional AON chemistries and delivery methods are currently in development. Dominic Wells presented data for peptide conjugated PMO (Pip6a-PMO) studies in mdx mice. Unlike their non-conjugated counterparts, Pip6a-PMOs are able to induce relatively high levels of exon skipping in both skeletal muscle and heart. Repeated systemic injections with Pip6a-PMO improved specific force and normalized force drop after eccentric concentration of the tibialis anterior. Volker Straub presented the effects of Pip6a-PMOs on cardiac function as assessed by cardiac MRI in mdx mice. In untreated mdx mice heart function was already impaired at 6 months of age, as reflected by reduced left ventricle volume, reduced stroke volume, increased right ventricle volume and reduced ejection fraction. Results with Pip6a-PMO AON treated animals are pending, but given that clear differences are found between wild type and mdx mice, an improvement in cardiac function should be picked up.

Prisca Boisguerin presented data from AON uptake mechanism studies. She compared the uptake of a peptide conjugated PMO (Pip6a-PMO) in cultured cells to study the internalization mechanism. The Pip6a-PMO was added to the medium and internalized within hours, without impacting cell viability. More internalization was observed for myotubes than for myoblasts. Further studies revealed that this process is likely ATP- and caveolae mediated endocytosis dependent. The internalization process was slower in cultured cardiomyocytes.

Alessandra Ferlini presented data on biocompatible nanomaterials as vehicles for AON delivery. She revealed that upon intraperitoneal injections of low doses of 2OMePS AON loaded on nanoparticles, AONs persisted for a long period (up to 3 months) in many tissues. However, exon skipping levels were very low, perhaps due to limited release of AONs by the nanoparticles or because relatively low doses of AONs can be delivered through nanoparticles. Interestingly, alginate coated nanoparticles loaded with 2OMePS AONs allow oral administration. Using this system to orally deliver AON-loaded nanoparticles dystrophin restoration was observed in some fibers in the diaphragm and some of the smooth muscle cells of the intestine. In terms of biodistribution, intraperitoneally administered nanoparticles are distributed throughout the body. Elimination is incomplete and levels tend to accumulate. Clearance is mainly (98%)
through feces. Orally administered nanoparticles are completely eliminated by feces in a few days.

Additional modified AONs are currently being developed. Most of these compounds have so far only been tested in small scale preclinical mouse experiments. Manufacturing of the compounds should obviously allow up scaling to larger quantities of clinically grade compounds. Furthermore, as these are new chemistries or modifications of existing chemistries, bridging studies will be required for these compounds to be allowed a shorter development path by the regulators. Performing placebo-controlled trials for these compounds will however be challenging. For large patients groups it can be anticipated that most eligible patients will already be involved in trials testing regular PMOs or 2OMePS AONs, while for smaller patients groups the number of patients available prevents conduct of a well powered placebo-controlled trial.

7. Cardiac issues

Francesco Muntoni presented an analysis of cardiac issues in DMD patients. With improved ventilatory support more patients now die from heart pathology (~40%), and almost all patients (at least 90%) have cardiac symptoms. Cardiac problems arise earlier than usually assumed; some patients already show signs of cardiomyopathy at 10 year old, but these often remain undetected because the patients do not show symptoms due to their physical limitations. It is clear that early detection and treatment of cardiac issues results in a much better outcome [28] and this is reflected now in the recent standards of care for DMD.

The concern with exon skipping is that for 2OMePS and PMO AONs exon skipping in animal models is observed in skeletal muscle but to a much lesser extent in heart [29]. Thus muscle function may be improved, leading to a larger burden for the heart. In mdx mice it has been shown that this can exacerbate the cardiac issues [30]. All participants agreed that it is is good to be vigilant about this (and monitor heart function in treated patients), but that it should not prevent further development of exon skipping. In the future strategies for heart treatment may be combined with AON treatment, such as perindopril or PDE5 inhibitors. Indeed early treatment and careful monitoring of affected siblings in families affected by X-linked dilated cardiomyopathy (a condition in which essentially no dystrophin is present in the heart while high levels of a normal or BMD like protein is produced in skeletal muscle) is able to delay the requirement of cardiac transplant (or the death of the affected individuals) by 2 decades (Muntoni personal observation).

8. Patient perspective

Three patient representatives (Kathi Kinnett, Parent Project Muscular Dystrophy, USA, Elizabeth Vroom, Duchenne Parent Project, the Netherlands and United Parent Projects Muscular Dystrophy and Francesca Ceradini, Parent Project Onlus, Italy) attended the meeting. They stressed that the development of exon skipping will require a combined effort of Industry, clinicians, scientists, regulators and patient representatives. Communication and regular updates to the patients will be of key importance. In the current day a misinterpreted quote can easily go viral through Facebook or other social media. So it will be very important that the patient community (and especially patients involved in trials) receive frequent updates from a reliable source. Even when there are no updates (e.g. analyzing results) this should be communicated. Also the development process of compounds, especially the regulatory approval process is a black box for the patient community. More transparency would be appreciated.

Something that worries the patient organizations is that all current trials are conducted in younger boys, which risks exclusion of older boys from treatment should AONs at some point obtain market approval. Furthermore, the patient representatives stressed that upon market approval it should be ensured that the compounds become available in sufficient amounts and at a reasonable price. Gathering extensive data on efficacy during postmarketing studies will be crucial for reimbursement purposes.

Patients should be involved in trial design in an early stage, as they are the ones who can be very helpful in defining what meaningful outcome measures would be and also are the best ones suited to assess risk/benefit ratios.

9. Future outlook

These are exciting times for the AON-mediated exon skipping field. However, it is clear that combined efforts of all key stakeholders are needed to develop this approach for as many patients as possible.

Communication will be key and the patient organizations hope to obtain commitment from the companies involved in exon skipping to provide correct and timely updates (e.g. 3-4 times per year) to patient community representatives, who can then translate this information (if needed) and distribute it to the wider DMD patient community.

To develop treatments for rare disease patients new paradigms have to be developed in which data sharing between groups is standard, at least for placebo groups and for which publishing results (regardless of the outcome of the trial) is mandatory.

The current focus is on confirming whether exon skipping really does slow down disease progression in phase III trials. Once that has been achieved, hopefully this will pave the way for the development of additional AONs. Smaller trials will however critically depend on the availability of additional outcome measures that are...
meaningful for the patients, as well as MRI, upper limb function tests and serum biomarkers. Therefore the effort of the field should be on (further) developing these tools so that they are available when needed.

This Workshop was made possible thanks to the financial support of the European Neuromuscular Centre (ENMC) and ENMC main sponsors:

- Association Française contre les Myopathies (France).
- Deutsche Gesellschaft für Muskelkranke (Germany).
- Muscular Dystrophy Campaign (UK).
- Musikinderfonden (Denmark).
- Prinses Beatrix Spierfonds (The Netherlands).
- Schweizerische Stiftung für die Erforschung der Muskelkrankheiten (Switzerland).
- Telethon Foundation (Italy).
- Spierziekten Nederland (The Netherlands).

And associated members:

- Finnish Neuromuscular Association (Finland).

With a special thanks to the Duchenne Parent Project (the Netherlands), Duchenne Parent Project Onlus (Italy), and the Parent Project Muscular Dystrophy (USA) for their generous support to this workshop.

10. Workshop participants

Dr. Annemieke Aartsma-Rus, Leiden University Medical Center, The Netherlands.

Dr. Pavel Balabanov, European Medicines Agency, United Kingdom.

Dr. Prisca Boisguerin, Université Montpellier 2, Montpellier Cedex, France.

Dr. Giles Campion, Prosensa Therapeutics, Leiden, The Netherlands.

Dr. Francesca Ceradini, Italian Parent Project, Onlus, Italy.

Dr. Judith van Deutekom, Prosensa Therapeutics, Leiden, The Netherlands.

Prof. George Dickson, Biological Sciences, Royal Holloway College, London, United Kingdom.

Prof. Victor Dubowitz, UCL Institute of Child Health, London, United Kingdom.

Prof. Alessandra Ferlini, Department of Medical Sciences, University of Ferrara, Italy.

Prof. Nathalie Goemans, University Hospital Leuven Gasthuisberg, Leuven, Belgium.

Dr. Ed Kaye, Sarepta Therapeutics, Cambridge, MA, USA.

Mrs. Kathi Kinnett, DMD Parent Project Muscular Dystrophy, USA.

Dr. S. de Kimpe, Prosensa Therapeutics, Leiden, The Netherlands.

Mr. Art Levin, miRagen Therapeutics, Boulder, CO, USA.

Dr. Afrodiite Lourbakos, Prosensa Therapeutics, Leiden, The Netherlands.

Prof. Jerry Mendell, Nationwide Children’s Hospital, Columbus, OH, USA.

Prof. Francesco Muntoni, UCL Institute of Child Health, London, UK.

Prof. Gert-Jan van Ommen, Leiden University Medical Center, The Netherlands.

Prof. Terry Partridge, Children’s National Medical Center, Washington, DC, USA.

Dr. Peter Sazani, Sarepta Therapeutics, Cambridge, MA, USA.

Prof. Ulrike Schara, University of Essen, Germany.

Prof. Volker Straub, Institute of Genetic Medicine, Newcastle, UK.

Prof. Shinichi Takeda, National Institute of Neuroscience, Tokyo, Japan.

Prof. Jan Verschuuren, Leiden University Medical Center, The Netherlands.

Dr. Elizabeth Vroom, Duchenne Parent Project, Amsterdam, The Netherlands.

Prof. Dominic Wells, Royal Veterinary College, London, UK.

Prof. Steve Wilton, Australian Neuromuscular Research Institute, Perth, Australia.

Dr. Daniel Zollinger, ENMC.

References


