

Long-term Exon Skipping Studies With 2'-O-Methyl Phosphorothioate Antisense Oligonucleotides in Dystrophic Mouse Models

Christa L Tanganyika-de Winter¹, Hans Heemskerk¹, Tatyana G Karnaoukh², Maaïke van Putten¹, Sjeff J de Kimpe², Judith van Deutekom² and Annemieke Aartsma-Rus¹

Antisense-mediated exon skipping for Duchenne muscular dystrophy (DMD) is currently tested in phase 3 clinical trials. The aim of this approach is to modulate splicing by skipping a specific exon to reframe disrupted dystrophin transcripts, allowing the synthesis of a partly functional dystrophin protein. Studies in animal models allow detailed analysis of the pharmacokinetic and pharmacodynamic profile of antisense oligonucleotides (AONs). Here, we tested the safety and efficacy of subcutaneously administered 2'-O-methyl phosphorothioate AON at 200 mg/kg/week for up to 6 months in mouse models with varying levels of disease severity: *mdx* mice (mild phenotype) and *mdx* mice with one utrophin allele (*mdx/utrn*^{+/-}; more severe phenotype). Long-term treatment was well tolerated and exon skipping and dystrophin restoration confirmed for all animals. Notably, in the more severely affected *mdx/utrn*^{+/-} mice the therapeutic effect was larger: creatine kinase (CK) levels were more decreased and rotarod running time was more increased. This suggests that the *mdx/utrn*^{+/-} model may be a more suitable model to test potential therapies than the regular *mdx* mouse. Our results also indicate that long-term subcutaneous treatment in dystrophic mouse models with these AONs is safe and beneficial.

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Introduction

Antisense-mediated exon skipping has a lot of therapeutic potential for Duchenne muscular dystrophy (DMD), a severe, progressive neuromuscular disorder that affects one in 3,500 newborn boys.^{1,2} This disease is caused by mutations, mostly deletions of one or more exons, in the dystrophin-encoding *DMD* gene that disrupt the reading frame and give rise to prematurely truncated proteins that are non functional.³ Dystrophin is crucial for maintaining muscle fiber stability and lack of dystrophin leads to exercise-induced damage and continuous loss of muscle fibers and function and eventually premature death usually in the 3rd decade.⁴ The exon skipping approach aims to reframe the disrupted transcripts, allowing the production of internally deleted, but partially functional dystrophin proteins, as are found in Becker muscular dystrophy patients.² This can be achieved with antisense oligonucleotides (AONs), small pieces of modified RNA that specifically hybridize to a target exon, hide it from the splicing machinery causing it not to be included in the mRNA. This approach is mutation-specific, but since deletions cluster in a hotspot region, skipping some exons applies to larger groups of patients. Exon 51 skipping would be beneficial for the largest group of patients (13%)⁵ and is currently developed in clinical trials. Initial trials using intramuscular injections of AONs of two chemistries [PRO051/GSK 2402968,

a 2'-O-methyl phosphorothioate (2OMePS) and AVI-4658, a phosphorodiamidate morpholino oligomer (PMO)] resulted in local dystrophin restoration in the majority of muscle fibers at ~15–35% of wild-type levels.^{1,6,7} Both compounds have also been tested in systemic trials.^{8,9} For PRO051/GSK2402968 (2OMePS chemistry) 5 weekly subcutaneous injections at different doses was well tolerated and induced novel dystrophin expression was observed in 60–100% of muscle fibers in post-treatment biopsies of 10/12 DMD patients.⁹ Dystrophin levels increased in a dose-dependent manner to up to 15.6% of that in healthy muscle. All 12 patients were subsequently enrolled in an open label extension study receiving weekly doses of 6 mg/kg. After 12 weeks, a mean improvement of 35.2 m (±28.7) was observed in the 6-minute walk test (>65 meters in three patients).⁹ Although these results are very encouraging, they will need to be confirmed by blinded and placebo-controlled follow up studies, which are currently ongoing. Weekly intravenous injections of AVI-4658 (PMO chemistry) for 12 weeks were tested at doses up to 20 mg/kg.⁸ This revealed dystrophin restoration in seven patients, especially for the higher dose groups. In three very good responders up to 55% of fibers became dystrophin positive and up to 27% of wild-type levels were observed.

Due to AON, dystrophin mRNA transcript and protein turnover, lifelong repeated AON treatment will be required. Animal models have been instrumental in the optimization of systemic

¹Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands; ²Prosensa Therapeutics BV, Leiden, The Netherlands. Correspondence: Annemieke Aartsma-Rus, Department of Human Genetics, Postzone S4-P, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands. E-mail: a.m.rus@lumc.nl

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delivery of AONs and assessing their pharmacodynamic and pharmacokinetic properties.^{6,7,10–16} The dystrophin-negative *mdx* mouse model is most commonly used. However, the phenotype in *mdx* mice is less severe than in humans: mice have a more efficient regeneration, muscle function is only mildly impaired and lifespan nearly matches that of wild type mice.¹⁷ In *mdx* mice, antisense-mediated exon 23 skipping resulted in dystrophin restoration and improved muscle function.^{10,14,18} Due to the minimal phenotype, it is more difficult to establish functional efficacy in this model. In other animal models, such as dogs, loss of dystrophin leads to a severe phenotype that better resembles the human situation.^{13,19,20} However, dogs are 20–150 times heavier than mice, making systemic studies very expensive.

The mild phenotype in *mdx* mice might in part be due to overexpression of the dystrophin homologue utrophin. Indeed, mice that lack utrophin in addition to dystrophin (double knock-out mice, *mdx/utrn*^{-/-}) have a very severe phenotype, with a limited lifespan (generally 2–3 months).²¹ It has been possible to prolong the lifespan of these animals to >1 year using peptide-conjugated PMOs.²² However, due to the severity of this model, animals could only be rescued when treatment started before weaning at very high doses; while treatment that was initiated later, or treatment with lower doses had no effect.²² It has been reported that dystrophin-negative mice with a single utrophin allele (*mdx/utrn*^{+/-}) have an intermediate phenotype, with higher levels of fibrosis than *mdx* and more impaired respiratory function.^{23,24} Our own studies show that muscle function is also more impaired than in dystrophin-negative mice with two utrophin alleles.²⁵ Thus, this model might be more suitable than traditional *mdx* mice for therapeutic studies, as it would allow a better assessment of the effect of exon skipping on muscle function and quality.²⁶

Toward long-term clinical trials we here tested weekly treatment with 2OMePS AONs at a high dose for a period of 6 months in *mdx* and *mdx/utrn*^{+/-} mice. In both models treatment did induce exon skipping, restored dystrophin and improved muscle function and integrity, in the absence of toxic effects. The therapeutic effect was more pronounced in the *mdx/utrn*^{+/-} mice.

Results

AON uptake and exon in *mdx* and *mdx/utrn*^{+/-} mice

Exon 23 skipping would bypass the nonsense mutation present in the *Dmd* gene of the *mdx* and *mdx/utrn*^{+/-} mouse models. We here used 2OMePS AONs targeting the 5' splice, previously identified by the group of Steve Wilton.¹¹ For 2OMePS AONs, exon skipping after systemic AON treatment of *mdx* mice can be achieved with a dose of 50 mg/kg/week, but the optimal dose appears to be 200 mg/kg/week,¹⁴ which would correspond to a dose of 16 mg/kg in humans after correcting for differences in body surface area between small and large animals (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078932.pdf>).

Mdx and the more severe *mdx/utrn*^{+/-} mice were treated with weekly subcutaneous injections of 200 mg/kg AONs or saline for 24 weeks starting at 4 weeks of age ($n = 7$ per group).

Treatment was well tolerated by all mice and body weights were similar for saline and AON-treated animals over time (data not shown). Mice were sacrificed 10 days after the last treatment. AON uptake by skeletal muscles, heart, diaphragm, liver, spleen, and kidney was assessed by an AON-specific ligation-hybridization assay (Figure 1a). This revealed higher uptake by kidney for the *mdx/utrn*^{+/-} mice (P value <0.05), whereas AON tissue levels were comparable for diaphragm, gastrocnemius, tibialis anterior, heart, and liver.

Reverse transcription-PCR analysis of skeletal muscles, heart and diaphragm showed exon 23 skipping for all treated mice (Figure 1b), whereas no skipping was observed in saline-treated mice (data not shown). No significant differences were observed between skipping levels observed for *mdx* and *mdx/utrn*^{+/-} mice for any of the muscles analyzed (Figure 1c). Exon skipping levels in heart were much lower (~5%) than in the other muscles (~15%), which has been reported before for 2OMePS AONs^{14,27–29} and PMOs.^{10,28}

Exon skipping also resulted in dystrophin restoration as assessed by Western blot analysis (Figure 1d,e). Dystrophin levels in gastrocnemius muscles were comparable to those obtained after 3-month treatment with 2 weekly injections of 100 mg/kg in both *mdx* and *mdx/utrn*^{+/-} mice (2–3% of wild-type levels).

Long-term AON treatment leads to improved muscle quality and function

Plasma creatine kinase (CK) levels are a marker for muscle fiber integrity and were assessed on a weekly basis. CK levels in untreated *mdx/utrn*^{+/-} mice were higher over time than those of *mdx* mice (Figure 2a). Although the dystrophin levels observed in treated mice were modest, CK levels dropped very significantly in treated mice over time (P value <10⁻¹⁴ and 10⁻¹⁵, for *mdx* and *mdx/utrn*^{+/-}, respectively; Figure 2b,c). No difference was observed between treated *mdx* and *mdx/utrn*^{+/-} mice.

Muscle function was assessed weekly by grip strength and rotarod analysis. No significant difference was observed at any time point or over time in grip strength between the different models or between the treated and untreated groups (data not shown). Without treatment, the *mdx* mice clearly outperformed *mdx/utrn*^{+/-} mice in the rotarod analysis over time (P value <0.01, Figure 3a), corresponding to our previous results in younger mice.²⁵ No difference could be observed between treated and untreated *mdx* mice (Figure 3b), whereas *mdx/utrn*^{+/-}-treated mice performed significantly better than untreated mice (P value <0.01; Figure 3c).

Long-term treatment with 2OMePS AONs is well tolerated

As described above, long-term subcutaneous AON treatment did not result in any obvious changes in body weight during treatment. Plasma was collected at the end of treatment and organs harvested for toxicological analysis, which did not reveal any toxic effects. Liver weights did not differ between treated and untreated mice (Figure 4a) and bilirubin levels (a marker for liver damage/inflammation) were below the detection limit for all mice (data not shown). Alkaline phosphatase (ALP) is a marker for hepatobiliary

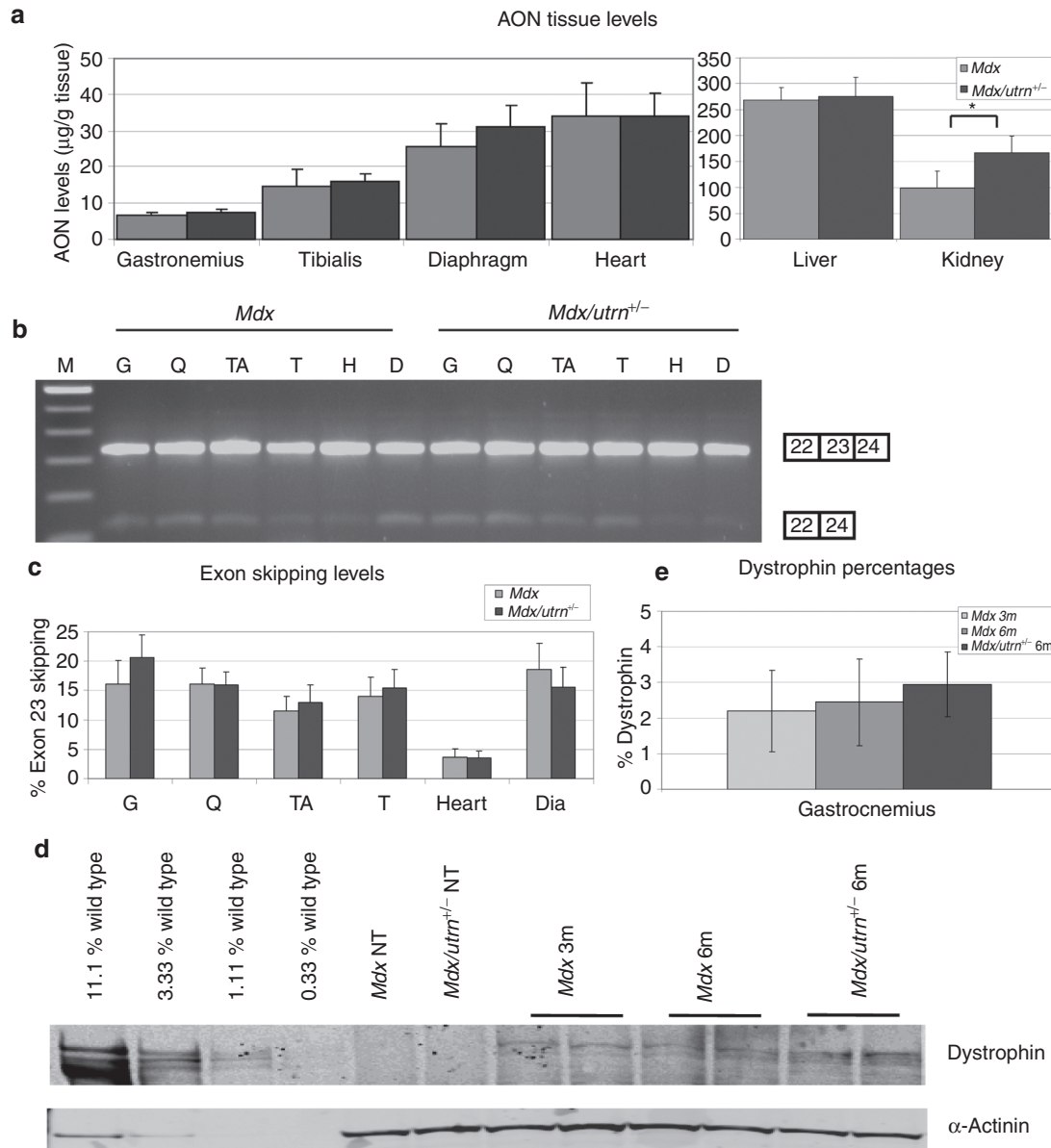


Figure 1 Biodistribution, RNA, and protein analysis of *mdx/utrn*^{+/-} and *mdx* mice treated for 6 months. (a) Biodistribution analysis. (b) Reverse transcription-PCR (RT-PCR) analysis of muscle samples from *mdx* and *mdx/utrn*^{+/-} mice. Exon 23 skipping could be confirmed for all samples. G is gastrocnemius, Q is quadriceps, TA is tibialis anterior, T is triceps, H is heart, D is diaphragm, C is negative control. The skip product is smaller than the wild-type fragment and thus binds less ethidium bromide, hampering visual estimation of skipping percentages. (c) Lab-chip analysis of exon skipping percentages. (d) Western blot analysis of gastrocnemius protein lysates of *mdx* mice treated for 3 or 6 months (3 m and 6 m, respectively) and *mdx/utrn*^{+/-} mice treated for 6 months. NT, not treated. α-Actinin staining was used to confirm equal loading. (e) Quantification of dystrophin levels for gastrocnemius. Error bars show the SD. **P* value <0.05.

function. ALP levels were increased in treated mice compared to untreated mice, although levels stayed within the normal range for all groups, but this difference was not significant (*P* value cut-off 0.01 after multiple testing correction) (Figure 4b). Glutamic oxaloacetic pyruvate transaminase and glutamate pyruvic transaminase are enzymes that are present in liver and muscle and leak into the bloodstream upon liver and muscle damage. Consequently, glutamic oxaloacetic pyruvate transaminase and glutamate pyruvic transaminase levels are elevated in dystrophic mouse models. Notably, levels in *mdx* mice are much higher than in

the more severely affected *mdx/utrn*^{+/-} mouse. This might be due to strain differences (C57/BL10 versus C57/BL6 backgrounds for *mdx* and *mdx/utrn*^{+/-}, respectively). Levels did not further increase for treated *mdx* mice, while a nonsignificant increase was observed for treated *mdx/utrn*^{+/-} mice (*P* value >0.01) (Figure 4c,d). The kidney weight was similar for all groups (Figure 4e), as were urea levels (a marker for kidney function) (Figure 4f). An increase in spleen weight could be observed for treated *mdx* mice, but this was not significant (Figure 4g). No difference in spleen weight was observed for *mdx/utrn*^{+/-} mice or in hemoglobin levels for both models

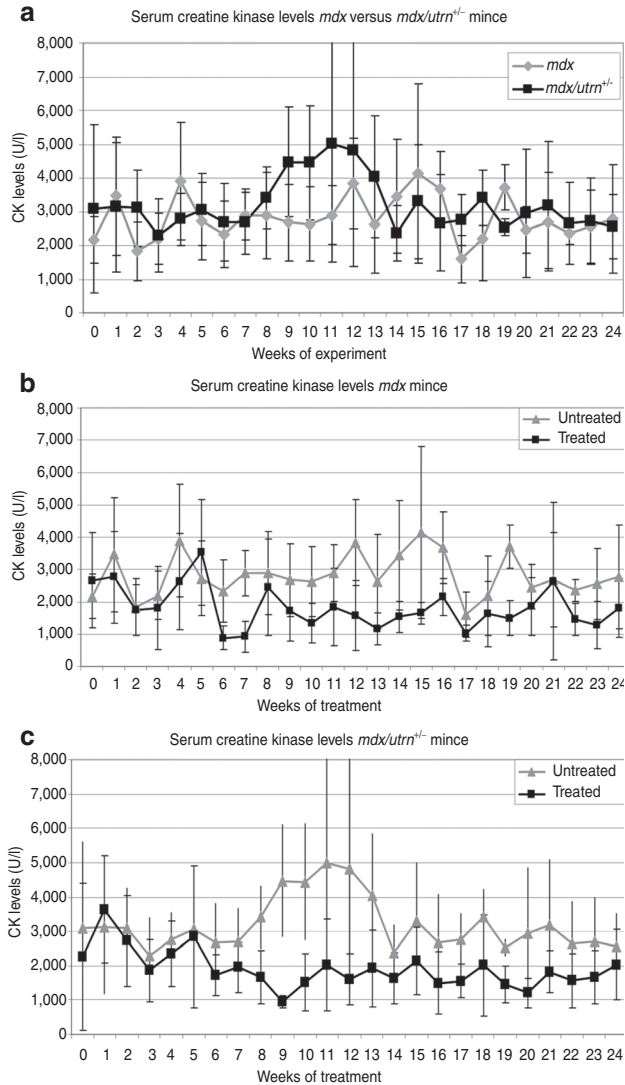


Figure 2 Plasma creatine kinases (CKs). (a) Saline-treated *mdx* versus *mdx/utrn^{+/-}* mice. CK levels are generally higher for *mdx/utrn^{+/-}* mice. (b) Saline versus antisense oligonucleotide (AON)-treated *mdx* mice. (c) Saline versus AON-treated *mdx/utrn^{+/-}* mice. For both models CK levels vary a lot, but starting at 6 weeks of treatment levels were lower in treated than untreated mice. In wild type mice, CK levels are below 500 U/l. Error bars show the SD.

(Figure 4h). These results confirm that 6-month treatment with a high dose of 20MePS AON is well tolerated.

Discussion

Antisense-mediated exon skipping is currently considered one of the most promising therapeutic approaches to treat DMD.¹ Due to AON, mRNA transcript and dystrophin protein turnover, repeated treatment will be required. AONs are very small and are therefore generally cleared by the kidney. Due to the PS backbone, 20MePS AONs will bind to serum proteins, which prevents renal clearance³⁰ and increases the half-life in serum and tissue. We have previously shown that 20MePS AONs are taken up efficiently by dystrophic muscle and that body wide

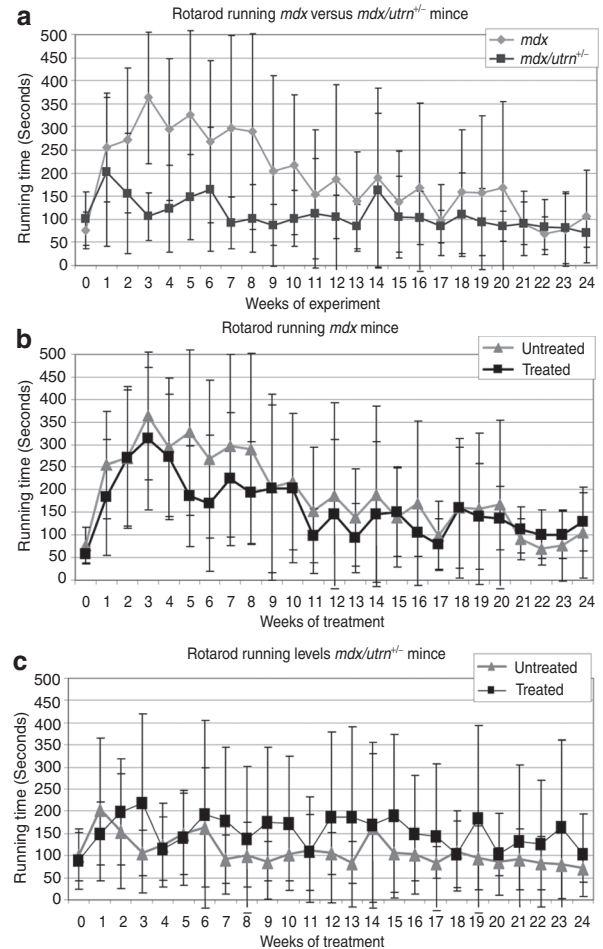


Figure 3 Rotarod running times. (a) *Mdx/utrn^{+/-}* perform less than *mdx* mice. (b) No difference could be observed for treated and untreated *mdx* mice. (c) Treated *mdx/utrn^{+/-}* clearly outperformed untreated *mdx/utrn^{+/-}* mice (P value <0.01). Error bars show the SD.

exon skipping and dystrophin restoration can be achieved after subcutaneous treatment for up to 3 months in *mdx* mice in the absence of toxic effects.^{14,27} We here tested 6-month treatment with high doses (200 mg/kg/week) in two different dystrophic mouse models, *i.e.*, the *mdx* and the slightly more severely affected *mdx/utrn^{+/-}* model. Compared to the 6 mg/kg/week dose used in clinical trials for GSK2404968, a 20MePS AON targeting exon 51, the 200 mg/kg/week dose required in mice seems high. However, a correction factor applies when translating doses between small and larger animals based on normalization to body surface area (<http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm078932.pdf>). When applying this correction factor, a dose of 16 mg/kg would be predicted for humans, which is in the same order of magnitude as the 6 mg/kg used in the trials.⁹ The slightly higher corrected dose for mice might be explained by a higher clearance rate for 20MePS AONs in mice, potential differences between pharmacokinetic and pharmacodynamic properties between exon 23 and exon 51 targeting AONs and the fact that a more efficient utrophin upregulation in mice leads to more stable muscle fibers and thus lower AON uptake.

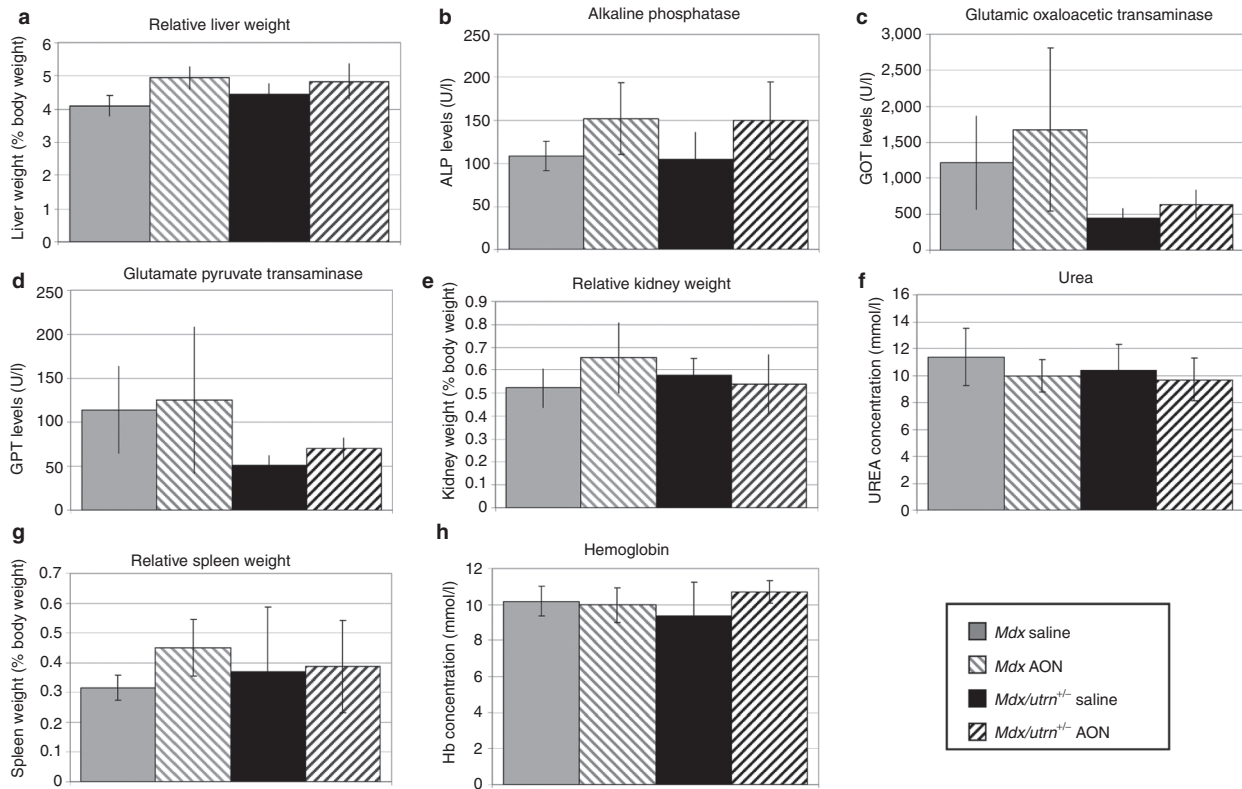


Figure 4 Toxicology analysis of treated and untreated mice. (a–d) Liver weight and liver damage and function markers. **(e–f)** Kidney weight and function marker. **(g)** Spleen weight. **(h)** Hemoglobin levels. Error bars show the SD.

We would like to propose the *mdx/utrn*^{+/-} model as an easier model than the *mdx/utrn*^{-/-} mouse, where breeding and husbandry is challenging. Still, the disease is more severe in the *mdx/utrn*^{+/-} model than in the traditionally used *mdx* mouse model. Higher levels of fibrosis and a more impaired respiratory function have been reported by others^{24,31} and we recently reported that the *mdx/utrn*^{+/-} model also performs worse than *mdx* mice in muscle function tests.²⁵ Thus, assessment of treatment effects on muscle function can be easier picked up in this model. Indeed, while in untreated animals CK levels and rotarod running times were significantly more affected in *mdx/utrn*^{+/-} mice, they were comparable for *mdx* and *mdx/utrn*^{+/-} after 6 months AON treatment. Thus, the same amount of dystrophin was able to achieve a larger therapeutic effect in the more severe *mdx/utrn*^{+/-} mice. While there is a clear benefit of these low dystrophin levels on muscle function and quality, it should be stressed that they do not fully normalize the phenotype. We have recently shown that levels of ~15–20% are probably needed for a more complete normalization of the phenotype in a mouse model with variable, low dystrophin levels due to skewed X-inactivation.³² Nevertheless, it is clear that even low levels of dystrophin already are beneficial.

Interestingly, we were unable to find a difference between treated and untreated *mdx* mice in rotarod running after 24-week treatment, while we have previously shown improvement in rotarod running in *mdx* mice after 8 weeks of AON treatment.¹⁴ As the analysis is done over time, a possible explanation for this discrepancy is the different duration of the experiments.

AON levels in muscles were comparable between the mouse models and highest in heart, while skipping levels were lowest for this tissue. However, the hybridization-ligation assay detects AON levels in the complete tissue and cannot discriminate between AON in the tissue and AON trapped in the interstitium. Based on the lower exon skipping levels observed in heart, we hypothesize more AONs are trapped in the interstitium for heart than skeletal muscles. Lower exon skipping levels in heart have been reported previously after systemic 2OMePS and PMO treatment,^{10,14–16,28,29,33,34} probably because the heart cells are not leaky like the skeletal muscle fibers and thus do not benefit from an enhanced uptake of AONs.^{14,28}

When exon skipping and dystrophin levels in *mdx* mice were compared to those obtained after 4, 8, and 12 weeks of treatment, no further accumulation was observed, while an increase had been observed previously up to 12 weeks.^{14,27} This might be explained by a different dosing regime that was adopted to reduce stress and handling for the mice. Rather than 2 weekly subcutaneous injections of 100 mg/kg, these mice received 1 weekly injection of 200 mg/kg. It has recently been shown that for PMOs multiple low doses are more efficacious than a single high dose.¹⁵ This may also be the case for 2OMePS AONs.

Even though dystrophin levels were quite low in treated mice, significant improvements could be observed in CK levels for both strains and in rotarod running time for the *mdx/utrn*^{+/-} mice. It has been reported previously that very low amounts of dystrophin have an effect on muscle quality.^{35,36}

This might be related to additional functions of the dystrophin protein in signal transduction and regulating miRNA levels.

Long-term treatment was well tolerated by all mice. There were some slight increases in some markers for liver function and liver damage, but none were significant. In addition, the differences were generally only found for one strain (either *mdx* or *mdx/utrn^{+/-}*). This suggests that 6-month treatment with high doses of 2OMePS AON by subcutaneous injections is well tolerated. However, patients will be treated even longer. AONs have thus far primarily been used to treat acute diseases and consequently, long-term safety data in humans is not yet available. Therefore, patients will have to be carefully monitored during long-term clinical trials that are currently ongoing for exon 51 skipping.

In conclusion, we show here that long-term subcutaneous treatment with 2OMePS AONs leads to exon skipping, dystrophin restoration, improved muscle quality and function in two DMD mouse models, in the absence of toxicity.

Materials and methods

All animal experiments were approved by the Animal Ethics Committee of the Leiden University Medical Center.

AON treatment of *mdx/utrn^{+/-}* and *mdx* mice. *Mdx/utrn^{+/-}* and *mdx* mice ($n = 7$ per group) were treated with weekly subcutaneous injections of 200 mg/kg M23D (+2–18) a 2'-O-methyl phosphorothioate AON described previously¹¹ or saline. Treatment started when mice were 4-week old and lasted for 24 weeks. Ten days after the last injection mice were sacrificed by cervical dislocation and muscles (gastrocnemius, quadriceps, tibialis anterior, triceps, heart, and diaphragm) and organs (liver, kidney, and spleen) were isolated and snap frozen in liquid nitrogen cooled 2-methylbutane and stored at -80°C .

Hybridization-ligation assay. The assay for measuring the AON concentration in tissue samples is based on a previously published hybridization-ligation assay.³⁷ All tissues and calibration curves of the analyzed AON were diluted 60 times in pooled control *mdx* mouse tissue lysates of the same type. The muscle samples were diluted 500 and 1,000 times, liver and kidney tissue 1,000 and 5,000 times. All analyses were performed in duplicate.

RNA isolation and reverse transcription-PCR. Muscles were minced in RNA-Bee (Campro Scientific, Veenendaal, the Netherlands) using MagNa Lyser green beads (Roche Diagnostics, Almere, the Netherlands) and the MagNa Lyser (Roche Diagnostics) according to the manufacturer's instructions. Total RNA was extracted and reverse transcription-PCR analysis was performed as described with a single round of PCR.³⁸ PCR products were visualized on 2% agarose gels and quantified with the Agilent 2100 Bioanalyzer (Agilent, Amstelveen, the Netherlands).

Protein extraction and western blot. Muscles were homogenized in treatment buffer (100 mmol/l Tris-HCl pH 6.8 and 20% sodium dodecyl sulfate) using MagNa Lyser green beads in the MagNa Lyser (Roche Diagnostics). Protein concentration was determined using the BCA protein assay kit

(Thermo Scientific, Etten Leur, the Netherlands), according to the manufacturer's instructions. Subsequently, the homogenate was completed to contain 75 mol/l Tris-HCl pH 6.8, 15% sodium dodecyl sulfate, 5% β -mercaptoethanol, 20% glycerol, and 0.001% bromophenol blue and boiled for 5 minutes. One hundred microgram was loaded on a 4–7% gradient polyacrylamide gel and run overnight at 4°C . Gels were blotted to nitrocellulose BA83 (Whatman, Schleicher & Schuell, Germany) for 6 hours at 4°C . Blots were blocked with 5% non-fat dried milk (Campina Melkunie, Maasdam, the Netherlands) in TBS followed by an overnight incubation with NCL-DYS1 (dilution 1:125; Novacastra, Leica Microsystems, Valkerswaard, the Netherlands) in TBS plus 0.05% Tween-20 to detect dystrophin. The fluorescent IRDye 800CW goat-anti-mouse IgG (dilution 1:5,000; Li-Cor, Lincoln, NE) was used as a secondary antibody. Blots were visualized and quantified with the Odyssey system and software (Li-Cor). α -Actinin (AB72592; Abcam, Cambridge, MA, dilution 1:7,500), was used as a loading control.

Plasma parameter assessment. Blood was collected weekly on Monday mornings in a Minicollect tube (0.8-ml Lithium Heparin Sep; Greiner Bio-One, Alphen aan den Rijn, the Netherlands) via a small cut at the end of the tail. CK levels were determined in plasma with Reflotron CK test strips in the Reflotron plus machine (Roche Diagnostics). Immediately before scarifying, blood was collected to assess plasma hemoglobin, ALP, urea, glutamic oxaloacetic transaminase, and glutamate pyruvate transaminase levels using the respective Reflotron test strips.

Functional analyses. Forelimb grip strength was assessed by means of a grip strength meter (Columbus Instruments, Columbus, OH). Mice were tested five times, with three consecutive measurements per trial (15 in total), and a 2-minute interval between trials. The three highest measured values were averaged to calculate the absolute strength, which was divided by the body weight in grams.

A rotarod test was performed weekly with the Rotarod (Ugo Basile, Comerio, Italy). Mice were placed on the rod that accelerated from 5 to 45 rotations per minute within 15 seconds. When a mouse ran for 500 seconds without falling from the rod, the test session was ended. Mice that fell off within 500 seconds were given a maximum of two more tries. The longest running time was used for analysis.

Statistical analysis. A two-tailed homoscedastic Student's *t*-test was performed in Microsoft Office Excel 2003 to compare AON tissue concentrations, exon skip levels, organ weights and plasma parameters between groups of mice. A *P* value <0.05 was considered significant for all tests, however for the analyses shown in **Figure 4** a Bonferroni correction was applied for multiple testing and a *P* value <0.01 was considered significant. To assess whether CK levels, forelimb grip strength or rotarod running times differed significantly over time between groups linear regression analysis was performed with statistical software in R (version R.2.11.1), using either mouse strain or treatment as variables. Data from week 0 were excluded from the analysis assessing a potential effect of treatment as this time point was before treatment. A *P* value <0.05 was considered significant.

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