Long term administration of antisense oligonucleotides into the paraspinal muscles of *mdx* mice reduces kyphosis

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Running Head
Antisense oligonucleotide induced reduction of kyphosis in *mdx*

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Abstract

The *mdx* mouse model of muscular dystrophy has a premature stop codon preventing production of dystrophin. This results in a progressive phenotype causing centronucleation of skeletal muscle fibers, muscle weakness and fibrosis and kyphosis. Antisense oligonucleotides alter RNA splicing to exclude the nonsense mutation, while still maintaining the open reading frame to produce a shorter, but partially functional dystrophin protein that should ameliorate the extent of pathology. The present study investigated the benefits of chronic treatment of *mdx* mice by once-monthly deep intramuscular injections of antisense oligonucleotides into paraspinal muscles. After 8 months of treatment, *mdx* mice had reduced development of kyphosis relative to untreated *mdx* mice, a benefit that was retained until completion of the study at 18 months of age (16 months of treatment). This was accompanied by reduced centronucleation in the latissimus dorsi and intercostals muscles and reduced fibrosis in the diaphragm and latissimus dorsi. These benefits were accompanied by a significant increase in dystrophin production. In conclusion, chronic antisense oligonucleotide treatment provides clear and ongoing benefits to paralumbar skeletal muscle, with associated marked reduction in kyphosis.

Keywords:
Duchenne muscular dystrophy, exon skipping, antisense oligonucleotides, *mdx* mouse, paraspinal muscles.
Introduction

The hereditary degenerative genetic disease Duchenne muscular dystrophy (DMD) results from mutations in the dystrophin gene that prematurely terminate dystrophin protein synthesis and cause loss of functional protein. Dystrophin is a component of the dystrophin-glycoprotein complex (DGC) that anchors the contractile machinery of myocytes to the myofibre membrane (6, 7). The absence of dystrophin leads to muscle cell weakness and deterioration, allegedly as a result of the breakdown in the normal architecture of the sarcolemma (6).

Antisense oligonucleotides (AOs) have shown promise as a potential treatment of DMD. AOs alter RNA splicing, allowing protein truncating mutations to be excised from the mature dystrophin mRNA and production of a shorter Becker muscular dystrophy (BMD)-like protein. As such, the severe DMD phenotype can be converted to a milder BMD phenotype. BMD patients have mutations in which the reading frame is typically retained but internal sections of the gene are deleted (8). This results in a partially functional dystrophin protein in which the N- and C-terminal ends are preserved (8).

Exon skipping has been successfully induced in cells derived from the \textit{mdx} mouse (12, 14, 15, 22) the golden retriever muscular dystrophy (GRMD) dog (16) and cultured myocytes from DMD patients (1, 19). No counteractive immune response has been found with repeated, current AO administration methods, which provides a significant benefit (12).

In \textit{mdx} mice, increased contractile forces have been reported 3 to 4 weeks after a single injection of AOs directly into the tibialis anterior muscles. In addition, protein expression has been induced by 2OMeAOs in 2 to 4 week old \textit{mdx} mice as well as in 6 month old mice (12). In that study dystrophin persisted for up to 2 months, although another group found that AO-induced dystrophin expression is much more transient, possibly due to both a loss of AO and protein turnover (21).

To date, there have been no studies reporting either chronic administration of AOs in mice or studies reporting their effects in very old \textit{mdx} mice, despite their skeletal muscles undergoing cycles of degeneration and regeneration, and showing pathology late in life closer to DMD dystrophinopathy (11, 17). There is a need to apply AOs to a range
of muscles over an extended period of time in order to assess the functional changes in those muscles and extend the therapeutic applicability of these techniques.

Thus, the aims of this study were to determine changes in the extent of kyphosis in AO-treated *mdx* mice and in the structure and function of intercostal, latissimus dorsi, and diaphragm muscles following 15 once monthly, deep intramuscular injections of AOs into paraspinal muscles.
Methods

Animals

Experiments were carried out on 3 groups of male mice (C57BL/10ScSn (C57): \textit{mdx} sham treated: \textit{mdx} AO treated). Mice were 2 months old at the beginning of the experiment and 18 months at the time of euthanasia. Animals were bred and housed at the University of Southern Queensland (USQ) Animal House, Toowoomba, Queensland. The mice were subjected to 12 hour day/night lighting cycles and given standard mouse chow and water \textit{ad libitum}. All experiments were conducted in accordance with guidelines of the National Health and Medical Research Council of Australia and were approved by the University of Southern Queensland Animal Ethics Committee.

Mice were anaesthetised monthly by subcutaneous injection of ketamine HCl 50 mg/kg (Ketamil, Troy Laboratories, Australia) and xylazine HCl 10 mg/kg (Ilium Xylazil-20, Troy Laboratories, Australia) subcutaneously prior to radiography and administration of AOs.

Radiography and determination of Kyphosis Index

Once anaesthetised, adhesive tape was used to lightly secure the mice to a radiographic cassette before being radiographed as described previously (10). The KI was calculated according to Laws & Hoey(10).

Antisense oligonucleotides

The hair over the dorsum was clipped, and the skin cleaned with ethanol prior to AO injections. The mice were positioned in ventral recumbency and the injection sites were located in the paraspinal muscles adjacent and parallel to the thoracolumbar vertebrae. The 30 g needles were placed deep into the longissimus dorsi muscles, orientated in a cranial direction and kept as flat as possible. Evidence from preliminary experiments using injections of dye showed that the majority of the injectate remained within the longissimus dorsi muscles, but a small amount spread within the dorsal portion of the latissimus dorsi muscle, or sometimes into adjacent intercostal muscles. These variations may be due to the needle depth, or occasionally a small volume of injectate could travel via fascial planes to other muscle regions. This variation in distribution was unlikely to cause concern, as it is highly probable that each muscle plays a contributory
role in the progression of kyphosis, and the latissimus and intercostal muscles were also evaluated in contractility studies. Three evenly spaced injections were administered on each side of the spine, to give a total of 6 µg of AO per mouse in a total volume of 40 µL.

The AO (M23D (+02-18)), consisting of 2'-O-methyl modified bases on a phosphorothioate backbone, was synthesised on an Expedite 8909 Nucleic Acid Synthesiser at Australian Neuromuscular Research Institute, University of Western Australia and transfection conditions (2:1 Lipofectin/AO ratio (w/w) in sterile 0.9% saline) were as described previously (15). Sham injections comprised the same volume of saline as previous studies had shown no difference between saline and Lipofectin sham groups. At the time of the final injection the AO-treated mdx had 2 µL of autoclaved histology marker dye (Wak-Chemie Medical, Germany) added to the 40 µL volume.

At the end of the procedure atipamezole 1mg/kg (Antisedan, Novartis Animal Health, Australia) was administered subcutaneously for the reversal of xylazine. Mice were kept warm and monitored until ambulatory.

**Contractility studies**

Mice were anaesthetised at 18 months of age using pentobarbitone sodium (Nembutal, Boehringer Ingelheim, Australia) at 70 mg/kg IP. The following muscles were dissected and placed into ice-cold Krebs buffer solution bubbled with carbogen (95% O₂/5% CO₂); a) a midcostal diaphragm strip from left midcostal hemi-diaphragm b) latissimus dorsi muscle and c) intercostal section comprising 4 ribs and their attached intercostal muscles (internal and external), extending from T8-12, adjacent and parallel to the longissimus dorsi muscle. Muscles from the left side were collected and stored for Western analysis and immunofluorescence. Contralateral muscles were utilised for contractility measurements and histology as described previously in Laws & Hoey (10).

**Histology**

Following contractility experiments, muscles were pinned onto cork at optimal length and then fixed sequentially in Telly’s fixative (formaldehyde, glacial acetic acid-ethanol fixative, 72 hours), Bouin’s solution (formaldehyde, glacial acetic acid-picric acid fixative, 24 hours) and 70 % ethanol, prior to paraffin embedding. Sections were cut at 10 µm and stained using 0.1 % w/v picrosirius red solution (Sirius Red F3B, Chroma
Dyes, Germany in saturated picric acid), a collagen specific stain. Fluorescent microscopy images were acquired using a digital camera (Q imaging Micropublisher 5.0 RTV) coupled to an epifluorescence microscope (Eclipse E600, Nikon, Japan). To grade collagen as a percentage of tissue area, four sections per tissue were photographed and analysed using AnalySIS (Soft Imaging System), then averaged. Additional 5 µm sections were stained with haematoxylin and eosin and viewed using bright field, with images acquired and analysed using the same equipment. Percentages of muscle fibres with centralised nuclei were determined in 200 fibres per muscle. All histological analysis was performed blinded to the strain of mouse or treatment.

**Protein extraction and Western blotting**

Protein was extracted from tissues and prepared for fractionation as previously described in Fletcher *et al.* (2006). Total protein was separated on SDS-PAGE using a NuPAGE precast 4-12% Bis-Tris polyacrylamide gel and MOPS SDS running buffer (Invitrogen). After electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane (Amersham Biosciences, Castle Hill, Australia) overnight at 290 mA at 18°C. Transfer was confirmed by staining the membrane with Ponceau S stain.

The membrane was blocked in 1x Tris-buffered saline-Tween 20 containing 5% skim milk powder and 0.1% Tween 20. For detection of dystrophin, the membrane was incubated with a 1:100 dilution of NCL-DYS2 monoclonal anti-dystrophin antibody (Novocastra, Newcastle upon Tyne, UK) for 2 hours at room temperature and afterwards for 1 hour in a 1:1000 dilution of peroxidase-conjugated antibody (rabbit anti-mouse IgG, Dakocytomation). The immunoreactive bands were visualised using ECL Plus and Hyperfilm ECL (Amersham Biosciences, Castle Hill, Australia). The film was analysed using Scion Image.

**Immunofluorescence**

Muscles were snap frozen in isopentane that had been pre-chilled using liquid nitrogen. Dystrophin was detected on 6 µm muscle sections as described previously in Fletcher *et al.* (2006), except that the Novocastra NCL-DYS2 primary antibody was used at a dilution of 1:25. Fluorescence was visualised and captured using a digital camera (Q
imaging Micropublisher 5.0 RTV) coupled to an epifluorescence microscope (Eclipse E600, Nikon, Japan).

Statistics

Results are expressed as means ± S.E. Responses between the mdx and control strain were analysed using Student’s unpaired t-tests or ANOVA for analysis of KI. $P < 0.05$ was considered statistically significant.
Results

Evaluation of long term AO administration

All mice tolerated the injection procedure well, with no adverse effects noted. There were no significant differences in final body weights between the three groups (C57 control: 30.4 ± 1.0g, \(mdx\) sham treated: 31.4 ± 0.9g, \(mdx\) AO treated: 31 ± 1.0g).

Kyphotic Index

KI as a measure of spinal deformity was similar for all three groups at 6 and 8 months of age. The AO-treated \(mdx\) showed a tendency to a greater KI than sham treated \(mdx\) as the mice aged (and hence less thoracic deformity) with this reaching statistical significance at 10, 12, 16 and 18 months of age (Fig 1 and 2). AO-treated \(mdx\) mice had a lower KI compared to C57 control mice from 10 to 18 months of age, however at 18 months, where a low KI is normally expected in \(mdx\) mice, there was no statistical significance between the \(mdx\) AO-treated and C57 mice.

Muscle contractility

There was a significant increase in weights of all \(mdx\) muscle preparations (sham and AO-treated) compared to wild type mice, however, there were no significant differences in muscle morphometry (optimal length (Lo), weight and width) between AO and sham treated \(mdx\) (data not shown). Whilst the \(mdx\) sham-treated latissimus dorsi, diaphragm and intercostal muscles were significantly weaker than C57 control muscles, there was no significant improvement in normalised twitch force or normalised tetanic forces in the latissimus dorsi, diaphragm and intercostal muscles of \(mdx\) mice treated with 1 \(\mu\)g AO (data not shown).
Histology

Of the four sham treated *mdx* muscles assessed, the diaphragm had the highest levels of fibrosis at 18 months old (*P*<0.05) as shown in Figures 3 and 4. There was also a significant amount of fibrosis in sham-treated *mdx* latissimus muscles (*P*<0.05) when compared to C57 mice. The AO treatment significantly reduced the fibrosis in diaphragm and latissimus muscles in *mdx* (Figures 3 and 4).

The characteristics of dystrophy, including variability in fibre size; centrally located nuclei, split and fused fibres, inflammatory cell infiltration and myocyte disarray, were apparent in all H&E stained sham-treated *mdx* muscles (Figure 5). These changes were more pronounced in the diaphragm and intercostal muscles than the latissimus and longissimus dorsi muscles. However, as illustrated in Figure 6, a lower incidence of centrally nucleated fibres was recorded in AO-treated *mdx* latissimus muscles (*P*<0.05) and intercostal muscles (*P*<0.01), despite the remaining large difference still present between the frequency of centrally nucleated myocytes in these treated *mdx* and C57 muscles (*P*<0.001).

Western blots & Immunofluorescence
Western blot analysis of C57 lumbar muscles showed full-length dystrophin at the predicted position (427 kDa), however dystrophin was not detected using this method in AO treated *mdx* muscles (data not shown). However, when longissimus muscles from AO treated *mdx* were examined more specifically using immunofluorescence, clusters of muscle fibres that were positive for dystrophin were observed (Figure 7). An average of 450 fibres per longissimus muscle were counted, with sham treated mice revealing 1.4% positive fibers (ie revertant fibers) with an average cluster size of 2.6, whereas longissimus muscles from *mdx* mice treated with 1 µg of AO had 2.9% positive fibers with an average cluster size of 4.5. To confirm the dose-dependency a further group of 6 age-matched *mdx* mice were injected with 2 µg of AO and euthanased after 4 weeks and these showed an average of 3.4% positive fibers (*P*<0.05 compared to sham treated) and an average cluster size of 8.2.
Discussion

Antisense oligonucleotide therapy of DMD is a promising approach to the disease in those boys whose genetic defect is amenable to forced alternative splicing. There are, however, a number of questions raised by this mode of therapy - including the safest and most efficacious route of administration, timing, long-term efficacy, and level of dystrophin expression required to ameliorate symptoms. This study sought to examine several of these issues in the mouse model of DMD.

This project examined the outcomes of monthly AO injections administered into the paraspinal muscles of mdx mice aged from 2-18 months of age. These ages were chosen as, although mdx show the most severe necrosis at weaning age, they continue to experience cycles of degeneration and regeneration throughout life, with gradual development of severe or moderate dystrophy in muscles such as the diaphragm, postural muscles and accessory respiratory muscles (11, 17). In addition, other important clinical features of DMD such as thoracolumbar deformity are seen in mdx mice by 18 months of age (10).

AO injections were well tolerated by the mice, and there was no apparent local swelling, loss of appetite or stiffness of gait following monthly treatments. The KI of the AO treated mice tended to be greater than sham injected mice (indicating less kyphosis) at all time points from 8-18 months of age with statistical differences between groups at 10, 12, 16 and 18 months of age. The KI of AO treated mdx mice tended to plateau from 12-18 months of age, but at 18 months of age was significantly indistinguishable from C57 mice. It can be concluded that 1µg AO injections into paraspinal muscles significantly attenuated thoracic deformity as indicated by an increase in the KI.

It was assumed that the increase in KI may be due to an improvement in muscle function in the mdx mice treated with AOs. However, there was no significant difference in force production in the latissimus dorsi, intercostal or diaphragm muscles from AO-treated mdx compared to sham-treated mdx. There are several possible explanations to account for this. The contribution of each muscle in causing or preventing kyphosis has not been clearly defined, so the role of the latissimus dorsi may not be significant, while the intercostal and diaphragm muscles are unlikely to contribute to reducing kyphosis. It
is highly feasible that the longissimus dorsi is the major muscle contributing to kyphosis, however, it is not possible to conduct whole muscle contractility studies on this muscle so quantitative assessment was not feasible. This point is particularly salient as the longissimus dorsi muscle received the majority of the AO injected. A third possible reason is for the lack of improvement in muscle strength may be an inadequate dosage or distribution of AO to the muscles in question. AO transfection is a local phenomenon, limited by concentration, the spread of injectate within tissues and degree of internalisation of AO by myocytes. Although evidence of dye was observed within other tissues such as latissimus dorsi or occasionally intercostal muscle regions, a dilution of effects could also result from this spread, i.e. there was less AO available to transfect fewer cells. It is feasible that a higher dose could have improved distribution or uptake resulting in greater dystrophin expression. Finally, this project examined function at one time point only (18 months old), and there may be a diminution of efficacy of AO therapy in dystrophic muscle with age, as satellite cell reserves and regenerative ability of muscle wanes and fibrosis advances. We could only speculate that although muscle strength may not have been improved in \textit{mdx} treated mice at this time point with this dose regime, the AO treatment was sufficient to prevent severe degeneration of muscle architecture and enough to make a significant impact on spinal curvature and the shape of the thoracic cavity.

Histology experiments revealed a small but statistically significant decrease in the percentage of fibrosis in the latissimus dorsi and the diaphragm muscles of AO-treated \textit{mdx} mice. Even though it was assumed that AO therapy would be unlikely to influence fibrosis in the diaphragm muscle, due to lack of direct diaphragm injections, it is possible that reduced contraction-induced injury has occurred as a consequence of the slightly increased thoracic area compared to that observed in the sham treated \textit{mdx}, as revealed by the increase in KI. This theory is in accordance with other studies on the \textit{mdx} diaphragm muscle which have stated that the increasing presence of fibrosis as the muscle degenerates may be an adaptive response to prevent overstretch injury (18). This was accompanied by a distribution of some of the ventilatory workload to the accessory respiratory muscles, which ultimately lead to their degeneration also (18). The AO treatment in this study has reduced spinal deformity which should in turn alleviate stress on the diaphragm. It is recognised that stretch of the \textit{mdx} diaphragm activatives NFkB
which in turn stimulates inflammatory and profibrotic cytokines (9). Conversely, it is possible that reduced diaphragmatic stress, alleviated by the reduced kyphosis may explain the reduced fibrosis observed in this study.

Evaluation of central nucleation in \textit{mdx} myocytes has demonstrated the attenuation of dystrophic pathology in other studies, including adeno-associated virus vector-mediated gene therapy (3, 20, 23) and stimulation of calcineurin signalling (2). The percentage decrease in centrally nucleated fibres of AO treated \textit{mdx} muscles in this study was statistically significant from untreated muscles, albeit only small. Thus it appears that a high amount of myofibre regeneration is still required to meet the demand of contraction-induced injury in a thoracic cavity that has a non-significant but slightly smaller average area than a C57 mouse.

It was not possible to demonstrate dystrophin expression in diaphragm, longissimus dorsi and latissimus dorsi muscle samples by Western blotting, despite the presence of strong dye staining to enable localisation of the sites of AO injection. This is most likely a result of low dosage of AO administered, as previously theorised. Immunohistochemical methods have proved to be more sensitive than Western blotting for demonstrating low level dystrophin induction following AO administration (13). This was supported by the presence of positive dystrophin fibres observed in the longissimus dorsi using immunofluorescence. While the number of dystrophin positive fibres observed by immunofluorescence was less than 10% of the muscle bundle, this appeared to correlate to the magnitude of the significant decrease in fibrosis of the latissimus dorsi and diaphragm, and the statistically significant decrease in centrally nucleated fibres in the latissimus dorsi and intercostals observed in the AO-treated \textit{mdx} mice. It has been reported that different muscle types are more amenable to AO-induced dystrophin synthesis. For example Lu et al.,(13) reported that after three weekly intravenous injections of AOs, gastrocnemius, intercostals and abdominal muscles expressed up to 5% normal dystrophin levels. Subsequent experiments in our laboratory have revealed that single injections of 1 \textmu g of AOs into tibialis anterior induced higher levels of dystrophin expression indicating that the longissimus dorsi may be less amenable to dystrophin expression compared to some other skeletal muscles. Similarly, subsequent
experiments with injections of 2µg AO induced synthesis of higher levels of dystrophin in longissimus dorsi indicating a dose-response relationship.

In conclusion, this study provides evidence that long term AO administration reduces muscle pathology in *mdx* mice, and over a period of 16 months of treatment significantly alters the dystrophic phenotype of kyphosis present in this strain. This benefit is despite low levels of dystrophin expression. However, even greater benefit may be obtained from using higher doses of AOs or using alternative chemistries such as phosphorodiamidate morpholino oligomers, which have a longer duration of action and appear to be more active *in vivo* (4, 5).
Figure Legends

**Fig 1.** Kyphotic Index as a measure of thoracolumbar deformity in AO treated *mdx* (n=6), sham (saline) treated *mdx* (n=5) and C57 (n=5) mice and additional untreated *mdx* and C57 mice (n=3). AO/Lipofectin complex or saline were injected intramuscularly into the paraspinal muscles once monthly from 2-18 months of age. KI was calculated from lateral radiographs. *P<0.05, **P<0.01 (comparing AO treated *mdx* and C57 mice), and ++P<0.01 (comparing AO treated *mdx* and sham injected *mdx*). There was evidence of reduced kyphosis in AO treated *mdx* compared to sham injected and untreated *mdx*, which reached statistical significance at 10, 12, 16 and 18 months of age.

**Fig 2.** Representative radiographs from 18 month old mice treated with either AO injections or sham (saline) injections into the paraspinal muscles. The yellow lines represent those constructed for the measurement of the Kyphotic Index (KI) = length of line ab/cd. A) C57 sham injected mouse. KI= 3.86 B) *mdx* sham injected. KI=3.05 C) *mdx* AO treated. KI=3.65. In the above examples kyphosis is more pronounced in the *mdx* sham injected mouse, resulting in a lower KI.

**Fig 3.** Fibrosis of latissimus dorsi, longissimus dorsi, diaphragm and intercostal muscles in control mice, sham treated *mdx* and AO treated *mdx* mice. Tissue fibrosis, as measured by analysis of picrosirius stained tissues was significantly greater in all *mdx* muscles compared to controls. For diaphragm and latissimus dorsi muscles there was significantly less fibrosis in AO treated *mdx* compared to sham treated *mdx*. *P<0.05, **P<0.01, P<0.001 (comparing AO treated *mdx* (n=6) and C57 mice (n=5)). +P<0.05 (comparing C57 and sham injected *mdx* (n=5)).

**Fig 4.** Latissimus dorsi (A, B, C), longissimus dorsi (D, E, F) diaphragm (G, H, I) and intercostals (J, K, L) muscle sections stained with picrosirius red. *mdx* mice (column 2) showed significantly greater fibrosis than control mice (column 1). In addition to the dense interstitial collagen network of dystrophin deficient muscle, there was also irregular myocyte size and fibre disarray. In AO treated mice there was a small, but statistically significant decrease in percentage fibrosis of the latissimus dorsi muscles (C) and diaphragm (I) muscles when compared to sham treated *mdx*. 
**Fig 5.** Incidence of muscle fibres with centrally located nuclei from control mice, AO treated *mdx* and sham treated *mdx* mice. The control mice characteristically showed peripheral nuclei (with their frequency here represented by the solid line adjacent to x-axis). There was a reduction in the percentage of central nucleation in AO treated latissimus and intercostal muscles. (P<0.001 when comparing *mdx* and C57 (n=5) mice). + P<0.05, ++ P<0.01 (comparing AO (n=6) and sham injected (n=5) *mdx*).

**Fig 6.** Representative H&E photomicrographs of latissimus dorsi (A, B, C), longissimus dorsi (D, E, F) diaphragm (G, H, I) and intercostals (J, K, L) muscle sections. Control mice muscle (column 1) had peripheral nuclei and little interstitial inflammation. Sham injected *mdx* mice (column 2) in contrast showed central nucleation and inflammatory cell infiltration typical of dystrophin deficient muscle. AO injected *mdx* latissimus dorsi (C) and intercostal (L) myocytes had significantly fewer central nuclei than sham treated *mdx* indicating a lower rate of degeneration and regeneration.

**Fig 7.** Representative immunofluorescence photomicrographs of longissimus dorsi muscle sections. Sections from C57 mice show typical dystrophin staining, whereas muscle sections from untreated *mdx* mice very few and isolated revertant fibers only. In contrast, *mdx* mice treated with 1 µg of AO typically exhibited small clusters of dystrophin positive fibers, while muscles from mice treated with 2 µg of AO showed significantly larger clusters of fibers.
References


Kyphotic Index of AO, sham injected and aging mice

- **C57, sham**
- **Mdx, sham**
- **Mdx, AO injected**

Age of mice (months)

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Note: Symbols indicate statistical significance:
- *p < 0.05
- **p < 0.01
- ***p < 0.001
Fibrosis, percentage of area of paraspinal and respiratory muscles
Frequency of centrally nucleated fibres in paraspinal and respiratory muscles

- Latissimus
- Longissimus
- Diaphragm
- Intercostals

Mdx, AO treated
Mdx, sham treated
C57
mdx 1 µg AO

mdx 2 µg AO

mdx negative control

C57 positive control