Chapter 2

A 3 months mild functional test regime does not affect disease parameters in young mdx mice

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Neuromuscular Disorders 2010; 20, 273-280

Abstract

To assess the effect of potential therapeutic agents in dystrophic mice it is useful to have a functional test regime that does not affect the natural disease progression of *mdx* mice with dystrophinopathy. We determined the effect of a 12 week test regime consisting of fore limb grip strength, rotarod analysis and two and four limb hanging wire tests on the disease progression of 4-week-old *mdx* mice. Mice performed the different functional tests on consecutive days on a weekly basis. No difference was found in serum creatine kinase levels between functionally active and sedentary mice. The percentage of fibrotic/necrotic areas assessed in a semi-automated way with colour deconvolution of skeletal muscles, heart and diaphragm did not vary within muscles or between groups, nor did the gene expression levels of disease-related genes. We conclude that this test regime may be suitable for short-term functional evaluation of therapeutic approaches in the *mdx* mouse.

Introduction

Duchenne Muscular Dystrophy (DMD) is an X-linked, severely progressive, muscle wasting neuromuscular disorder, which is caused by frame-shifting or nonsense mutations in the *DMD* gene. Translation of the disrupted reading frame results in the synthesis of truncated, non functional dystrophin protein (Hoffman et al. 1987). Intact dystrophin anchors the intracellular cytoskeleton of muscle fibers to the extracellular matrix and thereby protects the fibers from disruption during contraction. It also controls the calcium (Ca^{2+}) homeostasis, which is essential for normal fiber function (Blake et al. 2002). In dystrophin-negative muscles the Ca^{2+} homeostasis is disturbed due to an excessive influx through stress-activated channels and membrane tears. This leads to altered mitochondrial metabolism and down regulation of several mitochondrial genes, the activation of proteases, such as calpain, and the production of reactive oxygen species, resulting in more fiber damage and more Ca^{2+} uptake (Chen et al. 2000;Whitehead et al. 2006). This Ca^{2+} induced cascade leads to fiber necrosis, which attracts inflammatory cells that trigger fibrosis. Regeneration of fibers compensates for the lost muscle fibers, but eventually the regenerative capacity of the fibers becomes exhausted resulting in the replacement of healthy fibers by connective and adipose tissue (Deconinck and Dan 2007). In most patients, this causes profound muscle weakness leading to wheelchair dependency in the early teens and death due to respiratory and heart failure in their mid twenties.

The *mdx* mouse carries a nonsense mutation in exon 23 of the mouse *Dmd* gene (Sicinski et al. 1989). It is the most commonly used mouse to study the pathogenesis of DMD and to test putative therapeutic approaches (Grounds et al. 2008). Despite the similarity in gene defects, *mdx* mice are functionally less severely affected than DMD patients and their life expectancy is only slightly reduced compared to healthy mice. Muscle degeneration appears in cycles, the most pronounced acute phase lasts from 3 to 8 weeks of age, followed by relatively successful regeneration. In contrast to skeletal muscles, the diaphragm and tongue reflect the severe pathology found in DMD patients (Chamberlain et al. 2007). The less severe phenotype of *mdx* mice is thought to result from a combination of improved regeneration and, to some extent, functional compensation by the dystrophin homologue utrophin, although other yet unidentified factors may play a role as well (Blake et al. 2002;Perkins and Davies 2002).

Nonetheless, as a consequence of the genetic defect, *mdx* mouse muscle fibers cannot produce functional dystrophin resulting in fibers that are, like human DMD fibers, more vulnerable to contraction-induced injury and degeneration. Serum creatine kinase (CK) levels are elevated and the muscle fibers exhibit the same pathology resulting in fibrosis and necrosis in all

skeletal muscles including the heart in aging *mdx* mice (De Luca et al. 2008;Perkins and Davies 2002;Spurney et al. 2008).

Due to the pathology, transcription levels are altered for genes involved in immunological and tissue remodelling pathways. Microarray analyses of a series of dystrophic mice has resulted in several biomarkers that correlate with disease severity and progression, and that normalize upon therapeutic intervention ('t Hoen et al. 2006). These biomarkers are thus a useful tool to follow the natural course of the disease and monitor therapeutic effects over time.

Phenotypic severity in *mdx* mice can be exacerbated by exercise and eccentric contraction regimes (De Luca et al. 2008). The effect of a mild exercise regime on disease progression is less well understood. Several studies revealed beneficial effects, while other studies point towards harmful effects on disease progression (Dupont-Versteegden et al. 1994;Radley et al. 2008).

To determine whether genetic or pharmacologic treatment strategies are effective, more insight in the functional, histological and biomarker levels during the natural history of the disease in *mdx* mice is needed. To achieve this, a functional test regime that is not detrimental to the phenotype is needed. Therefore, we here assessed the effect of a 12 week functional test regime, consisting of four functional tests, on young *mdx* males. We monitored functional performance over time, and compared levels of previously identified fibrotic and immunologic RNA biomarkers between functionally challenged and unchallenged mice. In addition, we assessed the percentage of necrotic, fibrotic and regenerating cells using histological staining followed by a new semi-automatic analysis method based on the principle of colour deconvolution (Ruifrok et al. 2003). This analysis provides a more accurate, unbiased and faster method to analyze histopathology in whole muscle sections.

Materials and methods

Animal care

Mdx mice (C57BL/10ScSn-mdx/J) were bred at the animal facility of the LUMC. Mice were housed in individually ventilated cages with 12-h light–dark cycles. Standard mouse chow and water was given *ad libitum*. All experiments were approved by the Animal Experimental Commission (DEC) of the LUMC.

Functional tests

Four week old male *mdx* mice were randomly assigned to a functionally challenged or unchallenged group, each consisting of five mice. Functionally challenged mice underwent a 12 week test regime, consisting of four different functional tests, each addressing different muscle groups and/or coordination. Mice performed the four tests in the afternoon on consecutive days on a weekly basis (Supplementary Table 1). When possible, standardized operating procedures from the TREAT-NMD network were implemented (http://www.treat-nmd.edu/activities/treatment/html). Both groups were weighed at the beginning and end of each week.

Creatine kinase level analysis

Blood was collected weekly on Monday mornings from all mice in a Minicollect tube (0.8 ml Lithium Heparin Sep, Greiner bio-one, Austria) through a small cut at the end of the tail. CK levels were determined in sera with Reflotron CK test strips in the Reflotron plus machine (Roche diagnostics Ltd., UK).

Forelimb grip strength test

The forelimb grip strength test was performed with a home-made grid attached to an isometric force transducer that measures peak force of the forelimbs (Ugo Basile, Italy). Mice were suspended above the grid, which they instinctively grasped, and then pulled backwards. Mice were tested five times, with three consecutive measurements per trial (15 in total), and a two minute interval between trials. The three highest measured values were averaged to calculate absolute strength, which was divided by the body weight in grams. The degree of fatigue was measured by comparing the average of the first two and the last two trails as described previously (Connolly et al. 2001).

Rotarod

With the Rotarod (Ugo Basile, Italy) coordination, balance, muscle strength and condition was tested. Mice were placed on the rod that accelerated from 5 to 45 rotations per minute within 15 s. When a mouse ran for 500 s without falling from the rod, the test session was ended. Mice that fell off within 500 s were given a maximum of two more tries. The longest running time was used for analysis.

Two limb hanging wire test

Overall body coordination and strength was tested by a two limb hanging wire test. Mice were suspended above a metal cloth hanger secured 30 cm above a cage. The mouse was released a few seconds after instinctively grasping the wire with its forelimbs. Depending on the functional ability of the mouse, all limbs and the tail were used during a 10 min hanging session. Mice that fell down before the 10 min time limit were given two more tries. The longest hanging time was used for further analysis.

Four limb hanging wire test

Mice were placed on a grid where it stood using all four limbs. Subsequently, the grid was turned upside down 15 cm above a cage. Mice that fell down before the 10 min time limit were given two more tries. The longest hanging time was used for further analysis.

Histological examination

Mice were euthanized by cervical dislocation at an age of 16 weeks. Quadriceps, gastrocnemius, tibialis anterior, triceps, heart and diaphragm muscles were dissected and snap frozen in 2-methylbutane (Sigma–Aldrich, The Netherlands) cooled in liquid nitrogen. Sections of 8 µm were cut with a Shandon cryotome (Thermo Fisher Scientific Co., Pittsburgh, PA, USA) on Superfrost Plus slides (Thermo Fisher Scientific, Menzel-Gläser, Germany) along the entire length of the muscle with an interval of 240 µm between the sections. The excess tissue belonging to the 240 µm intervals was collected in MagNa Lyser Green Beads tubes (Roche diagnostics Ltd., UK) for total RNA isolation. Sections were fixed for five minutes with ice-cold acetone and stained with Harris haematoxylin and eosin (H&E) (Sigma–Aldrich, The Netherlands) according to conventional histological procedures. The diaphragm was stained with the Masson's trichrome staining (Sigma–Aldrich, The Netherlands). Sections were examined with a light microscope (LeicaDMLB, Leica Microsystems, The Netherlands) at a 2.5 times magnification at five locations in the muscle. Images were captured with a Leica DC500 camera and Leica IM50 software (Leica Microsystems, The Netherlands). Blending and background correction was performed with Adobe Photoshop CS3 version 10.0.1. Freely available ImageJ software with the

haematoxylin/eosin (H&E) colour deconvolution plugin (Rasband, W.S., ImageJ, US National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997–2008) was used to determine the fibrotic/necrotic percentage of the entire cross section. This plugin provides a number of "built in" stain vectors to provide an accurate stain separation of the green, blue and red component, which is based on the primary and secondary three-dimensional colour space (RGB and CYM) (http://www.dentistry.bham.ac.uk/landinig/software/cdeconv/cdeconv.html). Based on this linear description, ImageJ plugins have been created for different histological stainings (Ruifrok et al. 2003). The total tissue area was determined on the original picture while the area of healthy cells was determined using ImageJ's automatic threshold on the eosin component, obtained after H&E colour deconvolution. For the diaphragm, (Masson's trichrome staining) the haematox component of this plugin was used. This allowed calculation of the percentage of fibrosis/necrosis ((whole area minus healthy area) divided by whole area).

Biomarker analysis

Total RNA was isolated using RNA-Bee (Tel-Test, Bio-Connect, Huissen, The Netherlands) and purified with the NucleoSpin RNA II kit according to the manufacturer's instructions including a DNAse digestion (Bioke, The Netherlands). The RNA concentration was measured on a Nanodrop (Nanodrop Technologies, DE, USA) and integrity was checked with a total RNA nano assay on a labchip assay (Agilent, The Netherlands). cDNA was synthesised with random hexamer primers and gene expression levels were determined for *CD68*, *Lgals3* (influx of macrophages and immune cells), *Tnnt2*, *MyoG* (regeneration), *Bgn* (extra cellular matrix) *Nox2*, *Nox4*, *Lox* (fibrotic), *GLUT 4* and *PGC-1α* (exercise induced genes) by Syber Green based Real Time qPCR (95 °C 10 s, 60 °C 30 s, 72 °C 20 s 45 cycli and melting) on the Roche Lightcycler 480 (Roche diagnostics Ltd., UK). *GAPDH* was used as a reference gene, since the expression of this gene did not differ between different muscles or over time. The C_p values were obtained with the second derivative maximum method and analyzed with genex (http://www.gene-quantification.com/download.html).

Statistics

Statistical analyses were performed with statistical software in the R package 2.5.1 (http://www.Rpackage.org). The two-tailed homoscedastic student's *t*-test was conducted for comparison between the groups on the weight, CK levels, histological and gene expression data. $P<0.05$ was considered significant for all tests, however to the gene expression data a Bonferroni correction was applied to correct for multiple testing, therefore a $P<0.01$ was considered significant. The Spearman correlation test, which is not affected by differences in the distribution of values for the different measured parameters, was used for comparisons of all data from both the functionally challenged and unchallenged mice. The data obtained in the last week of functional testing were used to determine a possible correlation between the functional performance and histology, gene expression and creatine kinase levels. For the correlation analysis $P<0.05$ was considered significant.

Results

Functional tests

A group of 5 four week old male *mdx* mice was subjected to a 12 week functional test regime that consisted of four different functional tests performed on consecutive days on a weekly basis. The functional tests consisted of grip strength analysis, Rotarod running and two and four limb hanging wire tests. Each functional test was performed only once a week at the same time in the

afternoon and addressed different muscle groups and/or coordination. A group of five unchallenged *mdx* males was used as a control group.

Body weight was assessed at the beginning and the end of every week. No significant differences were found for average body weight at any time point or over time between functionally challenged and unchallenged mice. Notably, in the functionally challenged mice, a clear trend of a fluctuating body weight was seen that can be related to their functional test regime. After each weekend, when no functional tests were performed, there was an increase in body weight while a decrease in body weight occurred during the week, when the functional tests were performed (Fig. 1A).

Serum CK levels were elevated compared to C57BL/10ScSnJ mice (<500 U/L, De Luca et al. 2008), but did not differ significantly between the functionally challenged and unchallenged mice at any time point or over time (Fig. 1B). For each functional test, mice performed badly in the first week of testing. This is partly a learning effect, but may also be due to the decreased strength that correlates to the re-, and degenerating cycles which are present at the age of 4 weeks. Forelimb grip strength corrected for body weight decreased gradually over time from 5 to 15 weeks of age. This result is in consensus with a decreased forelimb strength found in *mdx* mice (5–12 weeks of age) that did not perform other functional tests beside the grip strength test De Luca et al. 2008).

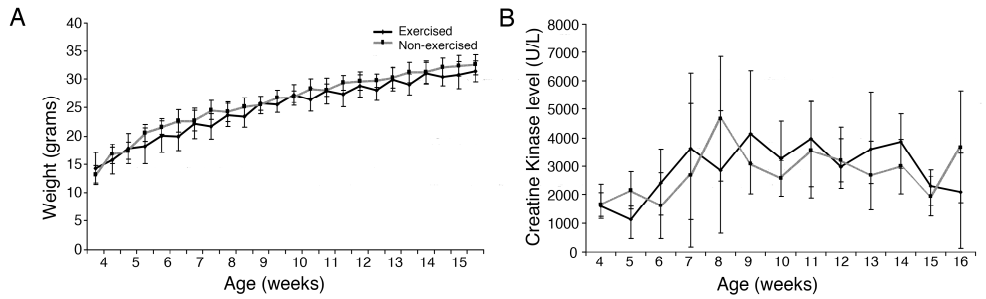

Fig. 1. Effect of the functional test regime on body weight and serum CK levels over time. (**A**) Body weight values of functionally challenged and unchallenged mice. Notably, a drop in weight can be observed during the week when tests were performed, while weight increases occurred during the weekends when no tests were performed.
(B) Serum CK levels of both groups over time. For both the body weight and serum creatine kinase levels, no significant difference was found between the two groups at any time point or over time. The error bars reflect the standard deviation of the average.

This decrease is mainly due to the increase in weight, as uncorrected strength remained virtually unchanged (Fig. 2A and B). No indications of fatigue were found at any time point (data not shown). On the Rotarod, the mice showed an average peak in their running time of 368 s at an age of seven weeks (i.e. after three weeks of testing). From then on, their performance declined to an average running time of around 200 s, which remained constant until the end of the test regime (Fig. 2C). This again is in agreement with previously reported data (Turgeman et al. 2008). Similar to the Rotarod peak performance, a peak at seven weeks was also observed with the two different hanging tests. However, the longest hanging time of both tests remained constant till an age of 12 weeks after which it dropped by 50% for both hanging tests (Fig. 2D and E), while the Rotarod performances dropped after the peak observed at an age of 7 weeks. For the Rotarod and both hanging wire tests no effect of body weight was found (data not shown).

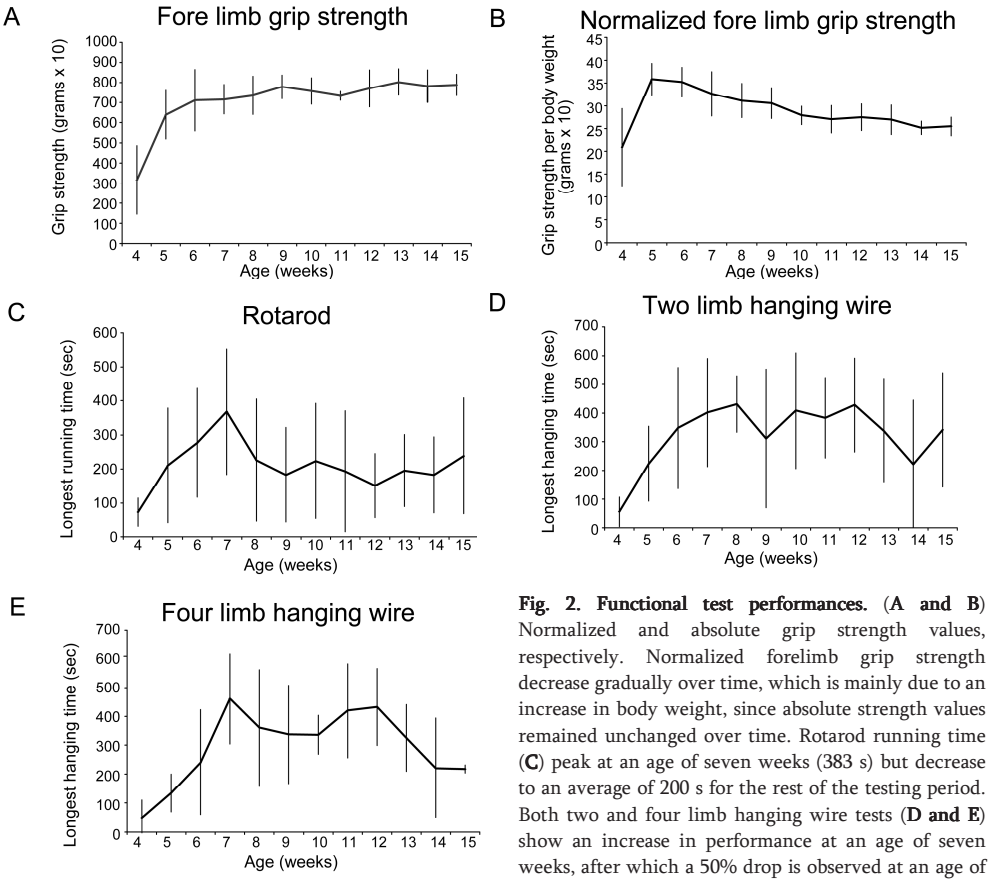

Fig. 2. Functional test performances. (**A and B**) Normalized and absolute grip strength values, respectively. Normalized forelimb grip strength decrease gradually over time, which is mainly due to an increase in body weight, since absolute strength values remained unchanged over time. Rotarod running time (**C**) peak at an age of seven weeks (383 s) but decrease to an average of 200 s for the rest of the testing period. Both two and four limb hanging wire tests (**D and E**) show an increase in performance at an age of seven weeks, after which a 50% drop is observed at an age of 14 weeks.

Histological examination

The quadriceps, gastrocnemius, tibialis anterior, triceps, heart and diaphragm were isolated from the functionally challenged and unchallenged mice at the age of 16 weeks. To determine levels of fibrosis, necrosis, connective tissue and recently regenerated tissue, a colour deconvolution based, semi-automatic analysis was set up on compiled pictures covering the entire cross sections. The haematoxylin/eosin plugin divides the picture in an eosin, haemotoxylin and background component. The eosin component represented the healthy area of the muscle examined. This area excluded fibrotic, necrotic, connective and recently regenerating tissue (Fig. 3).

In order to determine whether fibrotic/necrotic cells were equally distributed throughout the muscle (from tendon to tendon) the percentage of fibrotic/necrotic cells was determined at five levels along each muscle. For both the hind-, and forelimb muscle the proximal part was referred to as the beginning of the muscle (A) and the distal part as the end (E). The areas in between were referred to as B, C and D respectively. For the diaphragm, the dorsal part was designated the beginning (A) and the ventral part the end (E), while for the heart the cranial and caudal parts were beginning (A) and end (E), respectively. No significant differences in fibrotic/necrotic cell percentages were found between the five locations for any of the analyzed muscles (Fig. 4A).

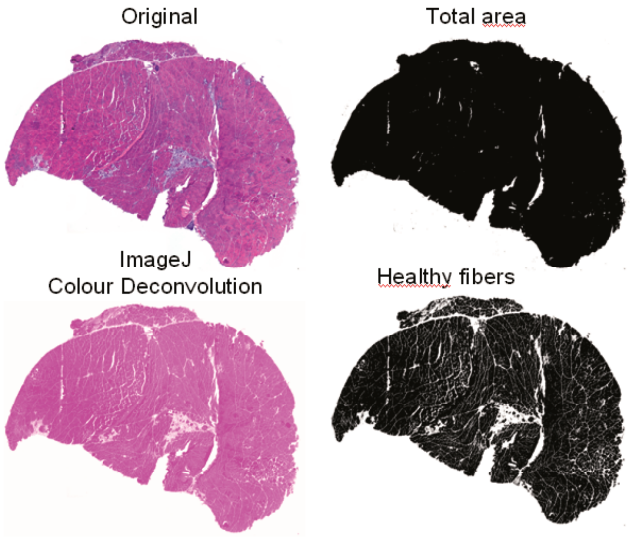

Fig. 3. Example of the semi-automatic histopathological determination using the colour deconvolution ImageJ plugin. The eosin component obtained with ImageJ after the haematoxylin/eosin colour deconvolution plugin represents the healthy area of the total cross section. Using this method, the percentage of necrotic, fibrotic, connective and recently regenerated tissue can be calculated from the total and the healthy fiber area.

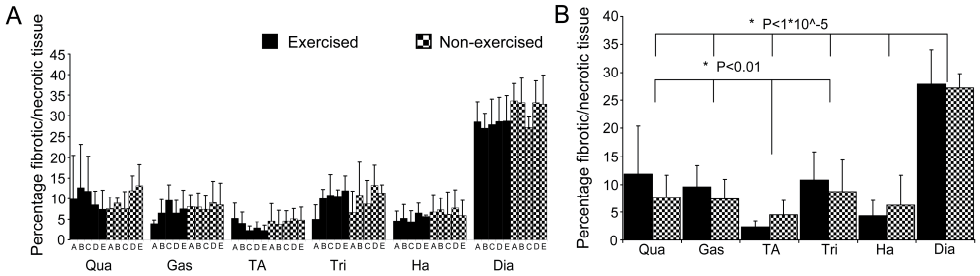

Fig. 4. Histopathology between different areas within a muscle (A) and comparison of histopathology between functionally challenged and unchallenged *mdx* mice for different muscles (B). (**A**) In four different skeletal muscles, the heart and diaphragm, the percentage of damage was determined at five locations from tendon to tendon. No significant difference was found between different areas within one muscle, or between functionally challenged and unchallenged mice. (**B**) A clear, significant difference in histopathological severity was found between different muscles; the tibialis anterior and the diaphragm were the least and most severely affected muscles respectively.

Therefore, for further comparisons only the middle section (C) of the muscle was analyzed. This revealed that the level of fibrosis and/or necrosis did not differ significantly between the functionally challenged and unchallenged mice in any of the muscles (Fig. 4B). This indicates that the functional test regime had no effect on tissue composition in *mdx* males. A clear difference in muscle damage severity was found between the different muscles. The tibialis anterior was significantly less affected (3.5%) than the gastrocnemius (8.5%), the quadriceps and the triceps (10% and 10.6%). The diaphragm was significantly more affected (30.3%) than all other muscles examined. The triceps was significantly more affected than the heart (5.9%). This is in accordance to previous studies based on several arbitrary fields (Turgeman et al. 2008) and an indication that the methodology used in the current study accurately detects changes in histological markers of muscle damage.

Biomarker analysis

The expression levels of several previously identified biomarkers that correlate with disease severity were assessed in the different muscles of the functionally challenged and unchallenged mice (Fig. 5) ('t Hoen et al. 2006;Baar et al. 2002;Spurney et al. 2009). The genes tested were *CD68*, *Lgals3* (influx of macrophages and immune cells), *Tnnt2*, *MyoG* (regeneration), *Bgn* (extra cellular matrix) *Nox2*, *Nox4*, *Lox* (fibrotic), *GLUT 4* and *PGC-1α* (exercise induced genes). When $P<0.05$ was considered significant, the expression of *Lox* differed between the groups in the diaphragm ($P = 0.027$), *Nox2* ($P = 0.038$) and *PGC-1α* ($P = 0.022$) in the tibialis anterior and *MyoG* ($P = 0.039$) and *GLUT 4* ($P = 0.010$) in the Triceps. However, after we conducted a Bonferroni correction, none of the tested genes were significantly differentially expressed between functionally challenged and unchallenged mice for any muscle.

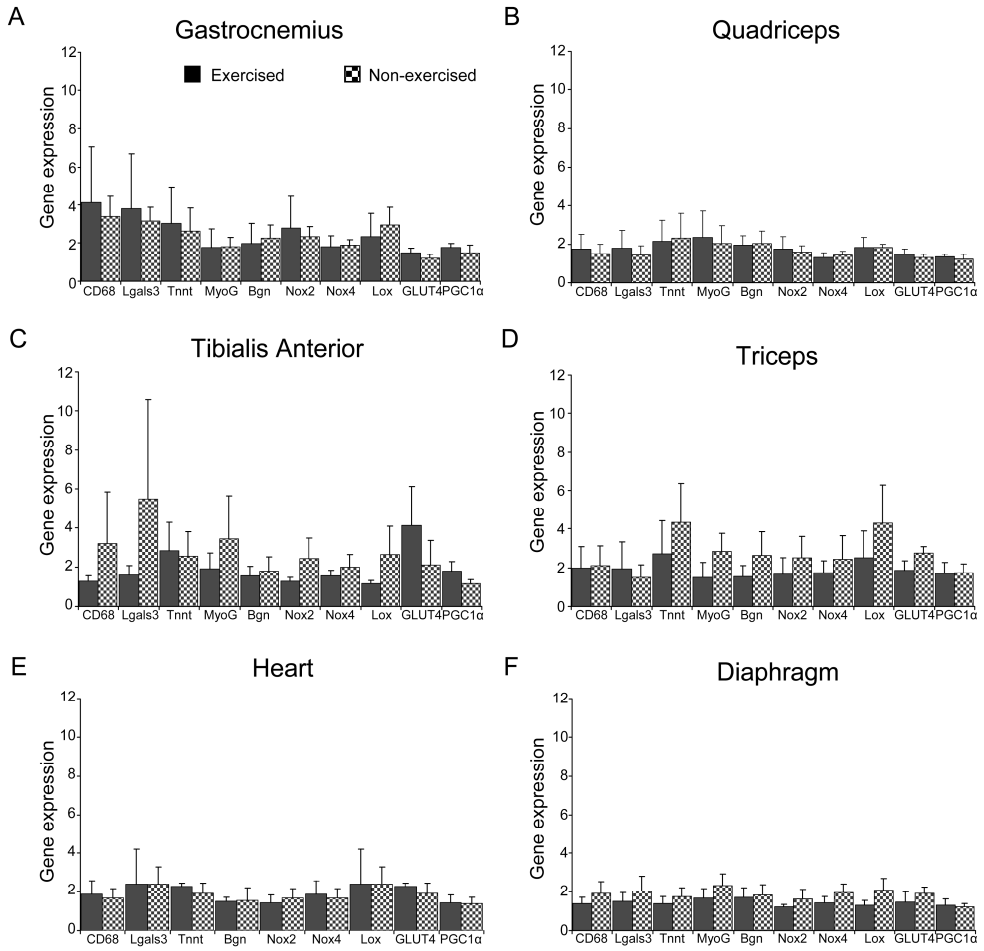

Fig. 5. Expression of genes involved in influx of macrophages and immune cells, regeneration, extracellular matrix stability, fibrosis and exercise. (**A**) Quadriceps, (**B**) gastrocnemius, (**C**) tibialis anterior, (B) triceps, (**E**) heart, (**F**) diaphragm. No significant difference was found between the functionally challenged and unchallenged mice for all the muscles examined.

Correlation between muscle function, histology and biomarker levels

Spearman correlation analysis was conducted to test for correlations between the functional performance, histological results and relative RNA levels of the genes examined in the current study. In addition we determined the correlation between genes (Table 1). No significant correlation was found between performance and histology, or between performance and serum CK levels. A positive correlation was observed between forelimb grip strength and the four limb hanging wire test ($P = 0.016$). Elevated serum CK levels correlated positively to the severity of fiber damage in the diaphragm ($P = 0.026$). A significant positive correlation was found between the percentage of fibrosis and both immunological genes (*CD68* $P = 0.011$ and *Lgals3* $P = 0.006$) in the tibialis anterior, indicating that the expression of these genes was increased in mice that had more fibrosis. In the gastrocnemius a significant positive correlation was found between histology and gene expression of *CD68* ($P = 0.013$), *MyoG* ($P = 0.006$), *Nox2* ($P = 0.033$) and *Lox* ($P = 0.003$). *GLUT 4* was positively correlated with histopathology in the quadriceps ($P = 0.042$) and *Bgn* was positively correlated with histopathology in the diaphragm (positive, $P = 0.017$) and *PGC-1α* (negative, $P = 0.021$). Almost all examined muscles showed a correlation between the immunological and fibrotic genes. In addition, correlations between genes involved in macrophage and immune cells influx, fibrosis and regenerative genes were found in some muscles. *Bgn* was correlated to immunologic and fibrotic genes in the quadriceps and triceps. Most correlations between genes were found in the quadriceps (21) and the fewest in the heart (6).

	Histology	CD68	Lgals3	Tnnt 2	MyoG	Bgn	Nox4	Lox	GLUT4	PGC-1α	Grip strength	Rotarod	2 LHW	4 LHW	CK
Histology															
CD68	TA G														
Lgals3	TA	TA G T D													
Tnnt 2		G T	G Q												
MyoG	TA G	TA G T D	TA G Q	G Q D											
Bgn	D	Q T H	Q T	Q T	Q T										
Nox2	G	TA G Q T D	TA Q	Q	G Q T D	Q T									
Nox4		Q H D		Q	G Q T D	Q H									
Lox	G	TA G Q T D	TA Q H		G T D	Q T	T D								
GLUT4	Q	**TA** D	**TA**	I D	I D	I		D							
PGC-1α	**D**			D											
Grip strength			D				G								
Rotarod										TA					
2 LHW								G							
4 LHW			D				G				TA G Q T H D				
CK	D					D				D					

Table 1 Significant correlations between histology, biomarkers and muscle function. Both tissue dependent positive and negative correlations were found between genes. Functional performance did not correlate to gene expression or histopathology, but some tests results did correlate to other tests. Fiber pathology did correlate to serum CK levels in the diaphragm. TA, tibialis anterior; G, gastrocnemius; Q, quadriceps; T, triceps; H, heart; D, diaphragm; LHW, limb hanging wire. Normal and bold abbreviations resemble positive and negative correlations, respectively. All correlations have P values <0.05.

Discussion

There is an increasing interest to gain more insight into the natural progression of the phenotype of the *mdx* mice on a functional, histological and biomarker level, since multiple studies aim to assess the efficacy of genetic or pharmacologic treatment strategies. Recently, effort was put into the standardization of pre-clinical testing in *mdx* mice with an emphasis on functional testing (Grounds et al. 2008;Spurney et al. 2009;Willmann et al. 2009). Due to inconsistencies in the experimental methods used, such as differences in outcomes measures, initiation age of the animals and exercise intensity and duration, discrepancies in the effects of exercise on disease progression are often seen in literature. Whether beneficial or detrimental effects will be found is largely influenced by the type of exercise. A delay in pathology progression can be found after voluntary exercise, such as swimming or wheel running (Carter et al. 1995;Dupont-Versteegden et al. 1994;Hayes and Williams 1998), while forced horizontal treadmill exercise results in muscle damage and is an often used protocol to enhance the pathogenic phenotype (De Luca et al.

2003;De Luca et al. 2008;Granchelli et al. 2000). In addition, age dependent effects have been found within an experimental protocol, where detrimental and beneficial effects were found in young (4 weeks) and old (16 weeks) animals, respectively (Faist et al. 2001). In pharmacological studies, treadmill exercise is often used to aggravate the pathogenic phenotype, after which grip strength analysis is used to monitor functional improvement (De Luca et al. 2008). Until now, the effect of a combination of several functional tests addressing different muscle groups and/or coordination on disease progression of *mdx* mice has not been studied.

We here assessed the effects of a 12 week functional test regime consisting of four functional tests, not including the treadmill, on disease progression. Our objective was to have a functional testing regime of which the outcome parameters would not interfere with the normal disease progression. We determined differences in histopathology and expression levels of previously identified fibrotic and immunologic RNA biomarkers between functionally challenged and unchallenged mice. The functional test regime used is intense and requires prolonged daily mouse handling. As this is an unavoidable part of functional testing, we actually compared the combination of functional testing and handling-induced stress to unchallenged mice that were handled only twice weekly for a short period to assess weight and CK. The fluctuations in body weight found in the present study might be caused by the functional test regime, however it cannot be ruled out that differences in handling time play a role as well.

Our functional test regime was chosen to assess which of the tests showed the lowest variation between mice over time, without interfering with disease pathology, thereby providing the most reliable outcome parameter(s). The current study included five male *mdx* mice per group, which is small. However, in a similar set up using dystrophic mice of different severities, we were able to pick up significant differences (unpublished data). To optimally use the *mdx* mouse for the efficient development of novel therapies to be used in humans, it is crucial to perform long-term blinded controlled studies in young and old mice, which thoroughly assess and compare the clinical phenotype of active versus placebo-treated animal cohorts. For these studies even longer term functional test regimes are needed.

The obtained grip strength and Rotarod data showed an age dependent performance decrease that is in accordance with previous data in sedentary *mdx* mice (mice that did not perform treadmill running prior to the functional test). The grip strength data obtained with the Ugo Basile meter are not comparable to those obtained by a Columbus meter by others. Preliminary data obtained with the Columbus meter in our *mdx* mice indicate that approximately two fold lower values are obtained with the Ugo Basile meter. Exercise, especially eccentric contraction, is known to directly cause an intensity dependent increase in serum CK level (Vilquin et al. 1998). Our functional test regime had no influence on the serum CK levels of the mice, which indicates that the functional test regime was mild. Carter et al. also found no effect on serum CK in mice that were exposed to voluntarily wheel running, while treadmill running is known to increase CK levels in *mdx* mice (Carter et al. 1995;De Luca et al. 2008). Both hanging wire test designs have not been reported before and the performances could therefore not directly be compared with results obtained by others. However, an age dependent de cline in hanging performance was earlier obtained with a different hanging wire test design (Golumbek et al. 2007).

Histological examination found no difference in pathology along the muscles. This indicates that the pathological features were equally distributed throughout the muscle. No difference in severity of muscle damage was found between the functionally challenged and unchallenged mice, which is in consensus with earlier data obtained after mild exercise (Carter et al. 1995). The percentage of damaged muscle differed between muscles, with the highest

percentage found in the diaphragm, which is known to be the most severely affected muscle in these mice (Ishizaki et al. 2008).

The here described semi-automatic procedure to determine histopathology provides an unbiased, accurate and fast method to determine the severity of histopathology in entire muscle cross sections. The main advantage of this methodology is that it provides an objective measure of fibrotic tissue but also of necrotic tissue, in contrast to other analysis in which only a collagen specific staining is used to determine the fibrosis percentage (Ishizaki et al. 2008). Furthermore, for this analysis the entire cross section of the muscle is used and not only a subsection, as was done in previous studies (Turgeman et al. 2008). Finally, the analysis software is freely available. This all together makes it an attractive method to determine histopathology in *mdx* mice.

In addition to comparing the functionally challenged and unchallenged mice on a histological level, we also looked at expression levels of several RNA biomarkers, but found no difference. *Lox* is known to be significantly increased in *mdx* mice compared to wild type mice (Spurney et al. 2009). A decreased *Lox* expression was expected in the heart upon a beneficial effect of exercise. However, no difference was found for this gene. This may be because the functional test regime was too mild to have an effect on the heart. Upon exercise the expression of genes like *PGC-1α* and *GLUT 4* were expected to increase, which is in contrast to our findings. However, the time gap between the last exercise and dissection was four days while in the study of Baar et al. this period was limited to 6 h (Baar et al. 2002). Therefore, by the time we analyzed the muscles, expression levels probably had already normalized. Comparison of gene expression levels between the different muscles was impossible due to the experimental design, which was aimed at comparing functionally challenged and unchallenged mice.

With the Spearman analysis, muscle dependent correlations between immunologic, fibrotic and regenerative genes were found. These correlations were in accordance to the pathogenic cellular events, in which necrotic fibers initiate immune responses and will be replaced by newly generated fibers (Deconinck and Dan 2007). Elevated serum CK levels correlated positively to the severity of fiber damage in the diaphragm. Due to a relative small sample size and high variation in biomarkers and other variables possible existing correlations might have been missed. A correlation between functional performance, histopathology and gene expression was not found. Possible correlations are likely to become undetectable due to high individual variations, but might be detected after a forced treadmill running protocol, which enhances the pathological phenotype.

Our findings indicate that our functional test regime is a useful tool to monitor muscle function and/or coordination in young *mdx* mice over time without accelerating the disease progression. Combined with our histological assay, this provides a convenient and accurate tool to functionally monitor the natural course of the disease in *mdx* mice.

Acknowledgement

We are grateful to J.M. Ross for her expert assistance with Image J analysis. This work is supported by the FP6 funded TREAT-NMD network of excellence and ZonNW.

Supplementary figure 1. Functional test regime.

Weight	•	•	•	•	•	•	•	•	•	•	•	•	•
CK	•	•	•	•	•	•	•	•	•	•	•	•	•
Grip strength	•	•	•	•	•	•	•	•	•	•	•	•	•
Rotarod	•	•	•	•	•	•	•	•	•	•	•	•	•
Hanging wire 2 limbs	•	•	•	•	•	•	•	•	•	•	•	•	•
Hanging wire 4 limbs	•	•	•	•	•	•	•	•	•	•	•	•	•
Age in weeks	4	5	6	7	8	9	10	11	12	13	14	15	16