Journal of Molecular and Cellular Cardiology 125 (2018) 29–38

Contents lists available at ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Voluntary exercise improves muscle function and does not exacerbate muscle and heart pathology in aged Duchenne muscular dystrophy mice

Bauke Kogelman[a], Kayleigh Putker[b], Margriet Hulsker[b], Christa Tanganyika-de Winter[b], Louise van der Weerd[a,b], Annemieke Aartsma-Rus[b], Maaike van Putten[b,*]

[a] Department of Radiology, Leiden University Medical Center, 2300RC Leiden, the Netherlands
[b] Department of Human Genetics, Leiden University Medical Center, 2300RC, Leiden, the Netherlands

ARTICLE INFO

Keywords:
Cardiomyopathy
Exercise
Low dystrophin levels
Magnetic resonance imaging
Heart

ABSTRACT

Duchenne muscular dystrophy is a severe muscle wasting disease, characterized by a severely reduced lifespan in which cardiomyopathy is one of the leading causes of death. Multiple therapies aiming at dystrophin restoration have been approved. It is anticipated that these therapies will maintain muscle function for longer and extend the ambulatory period, which in turn will increase the cardiac workload which could be detrimental for cardiac function. We investigated the effects of voluntary running exercise in combination with low dystrophin levels on function and pathology of skeletal muscle and heart. We divided 15.5-month old female *mdx* (no dystrophin), *mdx-Xist*$^{\Delta hs}$ (varying low dystrophin levels) and wild type mice (BL10-WT and *Xist*$^{\Delta hs}$-WT) to either a sedentary or voluntary wheel running regime and assessed muscle function at 17.5 months of age. Thereafter, a cardiac MRI was obtained, and muscle and heart histopathology were assessed. We show that voluntary exercise is beneficial to skeletal muscle and heart function in dystrophic mice while not affecting muscle pathology. Low amounts of dystrophin further improve skeletal muscle and cardiac function. These findings suggest that voluntary exercise may be beneficial for skeletal muscle and heart in DMD patients, especially in conjunction with low amounts of dystrophin.

1. Introduction

Duchenne muscular dystrophy (DMD) is a severe and progressive muscle wasting disease, caused by mutations in the *DMD* gene that prevent synthesis of functional dystrophin proteins. In muscle, dystrophin anchors the intracellular cytoskeleton to the extracellular matrix, thereby providing structural stability during contractions. Muscle fibres devoid of dystrophin are highly susceptible to injury and replaced by connective and adipose tissue upon exhaustion of the regenerative capacity of the muscle. Due to progressive muscle wasting, patients become wheelchair dependent in the 2nd decade of life and die before their 4th decade of life due to cardiorespiratory failure [1].

Because of the improved respiratory care, cardiac failure is now one of the main leading causes of death [2]. Already at six years of age, a quarter of DMD patients have some kind of cardiac involvement, while at the age of 18 years almost all DMD patients are affected [2,3]. One key characteristic of cardiomyopathy is the dilated left ventricle, found in the majority of older DMD patients [3–5], while studies on right ventricular function are less coherent [6–8]. Early detection is challenging, since cardiac involvement does not always result into clinical symptoms due to exercise intolerance resulting from skeletal muscle weakness.

Not only DMD patients, but also Becker muscular dystrophy (BMD) patients suffer from cardiac myopathy. BMD patients have in-frame mutations in the *DMD* gene leading to synthesis of reduced levels of truncated, but partially functional dystrophin proteins. Consequently, their muscle pathology is less severe than DMD patients. However, despite the expression of low dystrophin levels, about two thirds of BMD patients develop cardiomyopathy [9–11]. Similarly, DMD and BMD carriers, expressing 50% of dystrophin, also have an increased risk for cardiomyopathy [12,13].

Currently there are two therapeutic strategies approved aimed to

Abbreviations: DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy; H2, two limb hanging test; H4, four limb hanging test; CK, Creatine kinase

* Corresponding author at: Leiden University Medical Center, Department of Human Genetics, DMD Genetic Therapy Group, Einthovenweg 20, Building 2, Room S4-38, Postzone S4-P, P.O. Box 9600, 2300RC Leiden, the Netherlands.
E-mail addresses: b.kogelman@lumc.nl (B. Kogelman), k.putker@lumc.nl (K. Putker), hulskermargriet@yahoo.com (M. Hulsker), c.l.tanganyika-de_winter@lumc.nl (C. Tanganyika-de Winter), l.van_der_weerd@lumc.nl (L. van der Weerd), a.m.rus@lumc.nl (A. Aartsma-Rus), m.van_putten@lumc.nl (M. van Putten).

https://doi.org/10.1016/j.yjmcc.2018.10.008
Received 20 August 2018; Received in revised form 21 September 2018; Accepted 8 October 2018
Available online 16 October 2018

restore dystrophin expression in DMD patients; namely stop codon read-through therapy (Ataluren, PTC Therapeutics, conditionally approved by the European Medicines Agency) and exon skipping therapy (Eteplirsen, Sarepta, received accelerated approval from the Food and Drug Administration, US) [14–18]. Ongoing efforts try to optimize bioavailability and efficiency of genetic therapies in skeletal muscle and heart [19–21]. As yet however, the effects are primarily found in skeletal muscle, which could potentially extend the ambulatory period in DMD patients. It is anticipated that this will increase the workload for the heart. It is however difficult to predict the modulating effects of this increased activity on the heart; in healthy subjects exercise is beneficial for cardiac function [22], while for DMD patients this is largely unknown due to the early loss of ambulation. This warrants the need to obtain understanding of the effects of dystrophin expression and exercise on skeletal and cardiac muscle function.

The most frequently studied animal model for DMD is the *mdx* mouse, harbouring a spontaneous mutation in exon 23 of the murine *Dmd* gene which hampers dystrophin protein synthesis [23]. Skeletal muscle function is impaired in *mdx* mice. These mice also suffer from dilated cardiomyopathy and extensive cardiac fibrosis, which becomes apparent from the age of 6 months onwards and deteriorates with age [24–26]. The effect of exercise on cardiac function has been studied in *mdx* mice, showing mixed results. Some studies found improvement of cardiac function [27], while others did not find a modulating effect [28,29] or found a deteriorating effect of exercise on cardiac function [30,31]. The effect of exercise on skeletal muscle function in *mdx* mice is more conclusive. Forced treadmill exercise generally results in decreased muscle function and worsening of muscle pathology [32,33], whereas voluntary exercise generally results in improved muscle function [34–38].

In this study, we investigated the effects of voluntary running exercise and the expression of low dystrophin levels on skeletal muscle and heart function and pathology in 18-months-old wild type, *mdx* and *mdx-Xist*$^{\Delta hs}$ mice. At this age, *mdx* mice have already developed severe cardiomyopathy and we hypothesized that voluntary running could also in this advanced disease stage positively modulate heart and skeletal muscle function. *Mdx-Xist*$^{\Delta hs}$ mice express low dystrophin levels and were used to assess whether these levels could partly ameliorate heart pathology and whether this effect is altered by exercise. The *mdx-Xist*$^{\Delta hs}$ mice were generated by crossing *Xist*$^{\Delta hs}$ females (carrying a mutation in the promoter of the *Xist* gene, which coordinates X-inactivation, but with an intact *Dmd* gene) with *mdx* males (which have a normal *Xist* gene, but do not express dystrophin). In the female *mdx-Xist*$^{\Delta hs}$ pups, the X-chromosome expressing the mutated *Xist* gene, but intact *Dmd* gene, is preferentially inactivated, allowing expression of varying but low amounts of dystrophin in skeletal and cardiac muscle [24,39,40].

We show that voluntary exercise improves both skeletal and cardiac muscle function in dystrophic and wildtype mice, while having no deleterious effect on muscle integrity and quality. Expression of 3.13–50% dystrophin in skeletal muscle and 2.8–20.1% dystrophin in cardiac muscle further increases function of skeletal and cardiac muscles. These data implicate that voluntary exercise protocols are not deleterious, but beneficial for muscle function in mice.

2. Material and methods

2.1. Animal care

Mice were bred at the animal facility of the Leiden University Medical Center, where they were housed in individually ventilated cages (IVC) at 20.5 °C with 12-h dark-light cycles and given standard RM3 chow (SDS, Essex, UK) and water *ad libitum*. To generate *mdx-Xist*$^{\Delta hs}$ mice, *Xist*$^{\Delta hs}$ females (carrying a mutated *Xist* gene resulting in preferential inactivation of the X-chromosome) were crossed with C57BL/10ScSn-*Dmd*^{mdx}/J (*mdx*) males (carrying only a mutated *Dmd* gene). Their female *mdx-Xist*$^{\Delta hs}$ offspring consequently expressed low and varying amounts of dystrophin in skeletal and heart muscles due to non-random X-inactivation [39,40]. In this study we included: $n = 16$ *mdx*, $n = 48$ *mdx-Xist*$^{\Delta hs}$, n = 16 C57BL/10ScSnJ (BL10-WT), and n = 16 *Xist*$^{\Delta hs}$-WT females. Since only the female *mdx-Xist*$^{\Delta hs}$ mice express low dystrophin levels, analyses in the other mouse strains were also done in female mice to allow direct comparisons between the genotypes. All experiments were approved by the Animal Experimental Committee of the LUMC (permit #12208) and executed following EU-guidelines.

2.2. Voluntary exercise

Since we aimed to assess the effect of voluntary running on cardiomyopathy in an advanced stage of cardiomyopathy, we randomly allocated mice to either a voluntary exercise or sedentary group, at 15.5 months of age. Voluntary exercise consisted of an 8-week period where mice were individually housed in a cage equipped with a running wheel for 3 or 4 days alternating per week (in total mice spent 28 days with a running wheel). For the remaining time they were housed in IVC cages without a running wheel, similar to the sedentary group. The voluntary running wheel (circumference 77 cm, produced in-house) was equipped with two sensors placed at 180° from each other allowing detection of unique rotations. The rotation data of each individual wheel was automatically uploaded to a computer.

2.3. Functional testing

At 17.5 months of age, mice were subjected to two and four limb hanging tests to assess overall muscle function, condition and coordination. Tests were performed twice with one-week interval between the two tests. For the two limb hanging test mice had to hang on a metal wire (secured 30 cm above a cage with bedding, diameter 3 mm). Hereto mice, handled by the tail, were directed towards the wire which they grasped with their two forelimbs only. Upon release, the timer started and from that moment onwards, mice were allowed to use their hind limbs and tail if their physical abilities allowed them to do so. For the four limb hanging test, mice were placed on top of a metal grid which was subsequently turned upside down above a cage with bedding. In contrast to the two limb hanging test, mice were allowed to hold the grid with all four limbs from the start onwards, which could be an advantage for weaker mice. For both hanging tests, mice were given a maximum of three attempts to complete a 10-minute hanging session. The maximum hang time was used for analyses. Hanging times were not corrected for bodyweight. These tests were executed using the standard operation produces published by the TREAT-NMD Alliance (DMD_M.2.1.004 version 4.0 and DMD_M.2.1.005 version 2.0) [41].

2.4. Cardiac magnetic resonance imaging

At the age of 18 months, cardiac magnetic resonance imaging (MRI) was performed under general anaesthesia using isoflurane in a 1:1 mixture of oxygen and air (total flow: 0.6 L/min). Induction of anaesthesia was performed by 4% isoflurane and maintained at approximately 1–2% isoflurane. Respiration rate was used to monitor the anaesthesia depth and was kept between 50 and 80 respirations per minute. Cardiac MRI was obtained in prone position using a 7 Tesla Bruker PharmaScan® (Bruker BioSpin, Germany) equipped with a 370 mT/m gradient system, a 38 mm birdcage quadrature coil and ParaVision 5.1 software. To assess cardiac function short-axis images were acquired with the retrospectively-gated Intragate FLASH sequence with the following settings: 8–9 slices; field-of-view: 3.4 × 3.4 × 0.1 cm; image matrix size: 256 × 144; repetitions: 256; flip angle: 18°; echo time: 1.7 ms; repetition time: 6 ms; navigator echo position: in-slice; number of reconstructed frames: 18.

For every image, the *endo-* and epicardium were manually contoured for both the left and right ventricle in Mass (mass-dev_18feb2015) [42], an in-house developed software package. A single person contoured the ventricles, while a second person checked the quality of the contouring. In case of disagreement the contouring was adjusted to obtain consensus. End-systolic and end-diastolic cardiac phases were automatically determined and as quality criterion of the cardiac contouring a maximum mass difference of the left ventricle of 10% was allowed between both cardiac phases. End-systolic volume (ESV) and end-diastolic volume (EDV) were determined and heart rate (HR) was extracted from the IntraGate sequence. These parameters were used to calculate the stroke volume (SV = EDV − ESV), cardiac output (CO = SV × HR) and ejection fraction (EF = SV/EDV × 100%). All cardiac volume parameters were normalized to body mass. To assess cardiac hypertrophy, heart-to-body mass ratio was calculated by dividing heart mass by the body mass.

2.5. Creatine kinase level analysis

Immediately after the induction of general anaesthesia for MRI, blood obtained *via* an angled tail cut was collected in a Minicollect tube (0.8 mL Lithium Heparin Sep, Greiner bio-one, Austria) and stored in shaved ice upon plasma separation by centrifugation for 5 min at 13000 RPM (17979 RCF) at 4 °C. Creatine kinase (CK) levels were measured with Reflotron® CK test strips and a Reflotron® Plus device.

2.6. Histological examination

After MRI, mice were sacrificed by cervical dislocation and the quadriceps and heart were dissected and snap frozen in 2-methylbutane (Sigma Aldrich, the Netherlands) cooled in liquid nitrogen. Sections of 8 μm were made on Superfrost Plus slides (Thermo Fisher Scientific, Menzel-Gläser, Germany) with a Leica CM3050 S Research Cryostat (Leica Microsystems B.V., Amsterdam, The Netherlands) along the entire length of the muscle with an interval of 240 μm between the sections. Excess tissue was used for protein isolation.

Sections of the quadriceps were stained with Haematoxylin and Eosin staining according to a conventional protocol. Pictures were generated at 10 times magnification of the middle section of the muscle covering its entire surface. The colour deconvolution plugin of ImageJ software (Rasband W.S., ImageJ, U.S. National Institutes of Health, USA, http://rsb.info.nih.gov/ij/, 1997–2008) was used to separate healthy and unhealthy tissue (containing fibrosis, necrosis and inflammation) of the merged picture. The threshold tool was used to quantify both the healthy and whole muscle area, based on which the percentage of pathologic tissue was assessed.

Heart sections were stained with goat-anti-Collagen type 1 (dilution 1:100, 1310-01 Southern Biotech, USA), donkey-anti-goat Alexa594 (dilution 1:1000, Invitrogen, the Netherlands) and DAPI. A fluorescent microscope (Leica AF6000 or BZ-X700 fluorescent microscope (Keyence, Osaka, Japan)) was used to examine the sections at a 10 times magnification and images covering the entire middle section of the heart were captured with a Leica DC350FX or BZ-X700 snapshot camera. The percentage of fibrotic tissue and whole muscle area was assessed with the threshold tool of ImageJ software.

2.7. Dystrophin quantification by Western blot

The quadriceps and heart of *mdx-Xist*$^{\Delta hs}$ mice were homogenized and dystrophin levels quantified by Western blotting using the Odyssey system as described previously [43]. Blots were stained overnight for dystrophin with NCL-DYS1 (dilution 1:125, Novacastra, UK) and alpha-actinin with AB72592 (dilution 1:7500, Abcam, UK). The fluorescent IRDye 800CW goat-anti-mouse IgG (dilution 1:5000, Li-COR, USA) and IRDye 680LT donkey-anti-rabbit IgG (1:10,000, Li-COR, USA) were used as secondary antibodies for dystrophin and alpha-actinin respectively. The lowest concentration in the standard curve was 2.5%.

2.8. RNA isolation and gene expression analysis

Diaphragm muscles were placed in 1.4 mm Zirconium Beads pre-filled tubes (OPS Diagnostics, Lebanon, USA) and homogenized in TRIsure isolation reagent (Bioline, London, United Kingdom) using a MagNA Lyser (Roche Diagnostics). Total RNA was isolated using the TRIsure isolation method and purified by applying a NucleoSpin RNA II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction. From 500 ng of RNA, cDNA was synthesized using random N6 primers (Thermo fisher scientific) and Bioscript enzyme (GCBiotech, Alphen aan den Rijn, the Netherlands) according to the manufacturer's instructions. Quantitative PCR was performed in triplo per biological sample with the use of the LightCycler 480 and the ready to use SensiMix reagents (GCBiotech). The LinReg qPCR method was used for analyses of gene expression levels which were normalized to the housekeeping gene *Gapdh*. For this analysis, $n = 5$ *mdx*, BL10-WT and *Xist*$^{\Delta hs}$-WT mice were randomly selected and $n = 15$ *mdx-Xist*$^{\Delta hs}$ mice per exercise regime. Mice of the latter strain were selected based on dystrophin expression levels of the quadriceps to ensure that equal proportions of mice with low or higher dystrophin expression levels were included in each exercise group.

2.9. Statistics

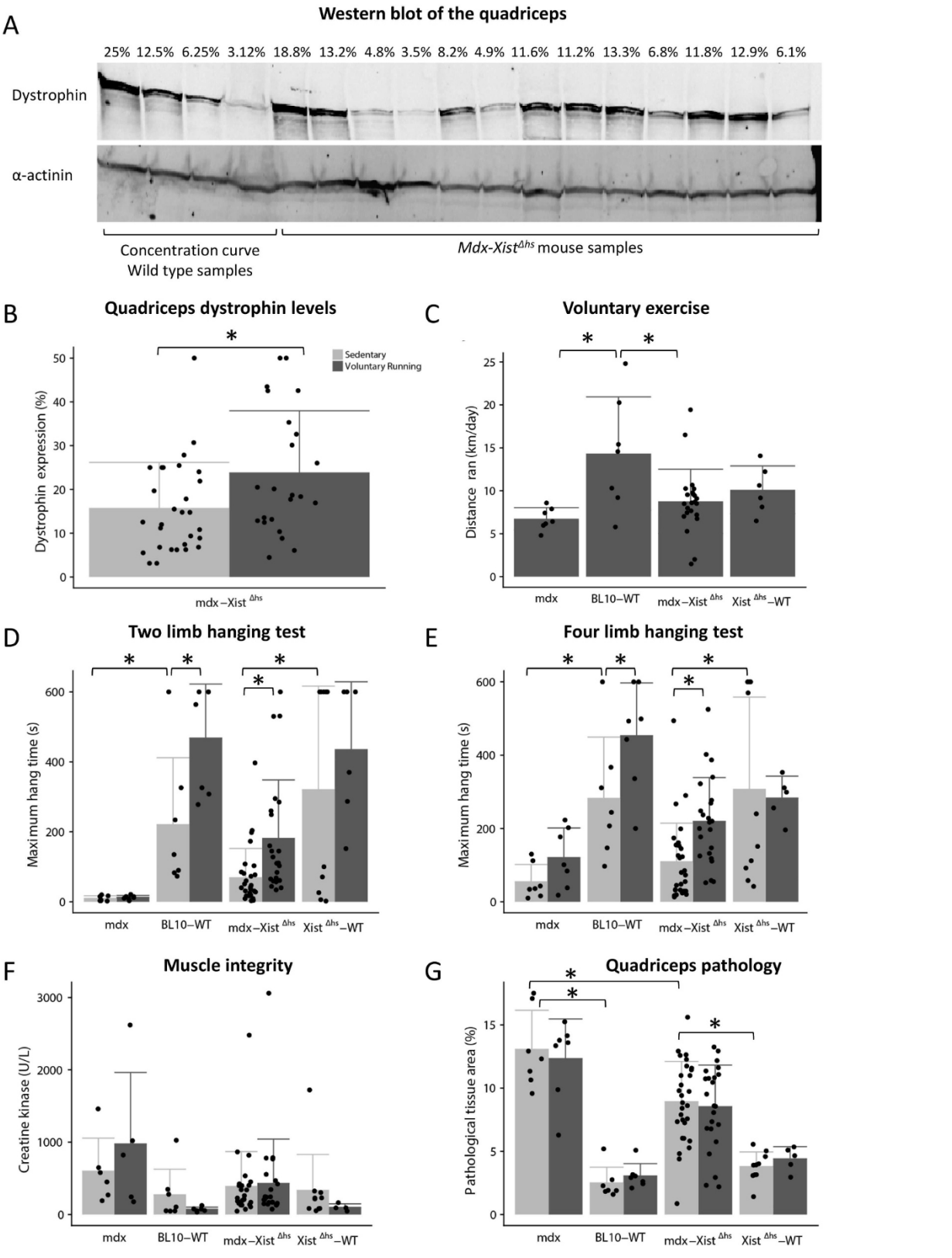
Statistical analysis was performed in R (3.3.0) using RStudio (1.0.143) [44,45] and Prism 4 (GraphPad Software, La Jolla, CA, USA). For comparison between strains a one-way ANOVA was used. To test the effect of voluntary running and strain on outcome measures a two-way ANOVA was used. A Fisher's least significant difference test was performed for 28 pair-wise combinations (4 strains, 2 exercise groups) in case the null-hypothesis in an ANOVA-test was rejected. Although this post-hoc test has less protection against a familywise error rate (FWER) compared to *e.g.* Tukey's range test or Bonferroni correction, we believe that it is the best trade-off between statistical power and controlling for the FWER, since only 7 of 28 (*mdx vs* BL10-WT and *mdx-Xist*$^{\Delta hs}$, *mdx-Xist*$^{\Delta hs}$ *vs Xist*$^{\Delta hs}$-WT and within each strain sedentary *vs* voluntary running) pairwise comparisons were of interest. Correlations between outcome measures were determined with Spearman's product-moment correlation test. Mice that did not sufficiently run were re-allocated to the sedentary group and this threshold was set by 1.5 times the interquartile range below the lower quartile assessed in all exercised mice (common outlier detection method in boxplots).

3. Results

3.1. Voluntary exercise improves skeletal muscle function and pathology

To study the effect of voluntary exercise and the expression of low amounts of dystrophin on skeletal muscle function we subjected 15-months-old female *mdx* (no dystrophin), *mdx-Xist*$^{\Delta hs}$ (low varying dystrophin levels) and BL10-WT and *Xist*$^{\Delta hs}$-WT (wild types) to voluntary running or sedentary groups. Voluntary running mice were provided with a running wheel for 28 days spread over a period of two months. The running distance for two *mdx-Xist*$^{\Delta hs}$ and three *Xist*$^{\Delta hs}$-WT mice was negligible (did not exceed the threshold of 837 m per day). Given that these mice performed well in the other muscle function tests and had good cardiac performance, we concluded that their unwillingness to participate in voluntary exercise was not due to pathology. Since they were voluntarily sedentary, we re-allocated them to the sedentary group, without violating the principle of randomization. The other mice ran significantly more than the threshold.

Mdx-Xist$^{\Delta hs}$ mice subjected to voluntary exercise had significantly higher dystrophin levels in their quadriceps than those assigned to the sedentary group (Fig. 1A-B, sedentary: average = 15.65% (range:



(caption on next page)

3.13–50%) *vs* exercise: average = 23.77% (range: 4.46–50%), $P < .01$). ANOVA analysis showed significant differences between mouse strains ($P < .01$) on the distance ran per day (Fig. 1C). *Mdx* mice ran significantly less compared to BL10-WT mice (*mdx* 6.75 *vs* BL10-WT 14.32 km/day, $P < .01$). Although low amounts of dystrophin in *mdx-Xist*$^{\Delta hs}$ mice did not significantly increase the distance ran per day compared to *mdx* mice (*mdx-Xist*$^{\Delta hs}$ 8.76 *vs mdx* 6.75 km/day, $P = .25$), it was also not decreased compared to wild type *Xist*$^{\Delta hs}$-WT mice (*mdx-Xist*$^{\Delta hs}$ 8.76 *vs Xist*$^{\Delta hs}$-WT 10.11 km/day, $P = .46$. *Xist*$^{\Delta hs}$-WT mice ran markedly less than the BL10-WT mice, which could

Fig. 1. Voluntary running and skeletal muscle function and pathology. One and two-way ANOVA analyses and Fisher's least significant difference post-hoc tests were performed. Significant differences (P < .05) are indicated with the asterisks sign. Bar graphs represent the mean and error bars the standard deviation. A) Representative Western blot of the quadriceps of *mdx-Xist*$^{\Delta hs}$ mice expressing low levels of dystrophin. To ensure equal loading in the concentration curve, wildtype protein lysates were diluted in *mdx* protein lysates. B) Mice allocated to the voluntary running group had significantly higher dystrophin levels in their quadriceps. C) 15.5-month-old mice ran 3–4 days a week for a 2-month period. *Mdx* mice ran less than BL10-WT mice and low amounts of dystrophin in *mdx-Xist*$^{\Delta hs}$ mice did not increase the distance ran. D) At 17.5 month of age muscle performance in the two limb hanging tests was impaired in sedentary *mdx* and *mdx-Xist*$^{\Delta hs}$ mice. Although in *mdx-Xist*$^{\Delta hs}$ mice maximum hang time was improved by seven-fold compared to *mdx* mice, this did not reach significance. Voluntary exercise significantly improved muscle function in *mdx-Xist*$^{\Delta hs}$ and BL10-WT mice. E) Muscle performance assessed with four limb hanging tests revealed impaired muscle function in the sedentary dystrophic mice. Although *mdx-Xist*$^{\Delta hs}$ had a two-fold improvement in hang time compared to *mdx* mice this did not reach significance. Hang time was more than doubled by exercise in *mdx* mice, but this did not reach significance. In *mdx-Xist*$^{\Delta hs}$ and BL10-WT mice exercise significantly improved muscle function. F + G) Although exercise generally improved muscle performance, it did not affect pathology measured by plasma creatine kinase levels and histopathology of the quadriceps muscle (the amount of fibrosis, necrosis and inflammation). Low dystrophin levels in *mdx-Xist*$^{\Delta hs}$ mice partly prevented pathology which was less severe compared to *mdx* mice, but still affected compared to both wildtype strains.

be due to lack of motivation. The amount of dystrophin in the quadriceps muscle and the distance ran did not correlate in *mdx-Xist*$^{\Delta hs}$ mice ($\rho = 0.35$, $P = .10$).

At 17.5 months of age, skeletal muscle function was assessed in all mice using two- and four-limb hanging tests (H2 and H4). In the sedentary group, muscle performance of *mdx* mice was impaired in both hanging tests compared to *mdx-Xist*$^{\Delta hs}$ and wild type mice, reaching significance for the latter strain (Fig. 1D + E). Low amounts of dystrophin in *mdx-Xist*$^{\Delta hs}$ mice improved hanging time by two to seven-fold compared to *mdx* mice, although this did not reach significance (H2: *mdx* 9.3 s *vs* *mdx-Xist*$^{\Delta hs}$ 68.6 s, $P = .37$; H4: *mdx* 54.4 s *vs* *mdx-Xist*$^{\Delta hs}$ 109.0 s, $P = .34$). In addition, *Xist*$^{\Delta hs}$-WT mice significantly outperformed *mdx-Xist*$^{\Delta hs}$ mice (H2: *mdx-Xist*$^{\Delta hs}$ 68.6 s *vs* *Xist*$^{\Delta hs}$-WT 320.5 s, $P < .01$; H4: *mdx-Xist*$^{\Delta hs}$ 109.0 s *vs* *Xist*$^{\Delta hs}$-WT 306.5 s, $P < .01$).

At strain level voluntary exercise improved muscle performance (H2 and H4: $P < .01$). Voluntary running in *mdx* mice did not affect hanging time in the H2-test, but more than doubled it in the H4-test, (H2: sed 9.3 s *vs* vol run 12.1 s, $P = .97$; H4: sed 54.4 s *vs* vol run 120.4 s, $P = .36$). In *mdx-Xist*$^{\Delta hs}$ mice, voluntary exercise improved muscle function in both hanging tests (H2: sed 68.6 s *vs* vol run 181.0 s, $P = .02$; H4: sed 109.0 s *vs* vol run 219.3 s, $P < .01$). It should be noted that part of this improvement could be due to the significantly higher dystrophin levels in the quadriceps of the exercised *mdx-Xist*$^{\Delta hs}$ mice. Voluntary exercise also improved hanging performance in BL10-WT mice (H2: sed 220.1 s *vs* vol run 468.0 s, $P < .01$; H4: sed 281.9 s *vs* vol run 453.0 s, $P = .02$), but not in *Xist*$^{\Delta hs}$-WT mice. At strain level the amount of voluntary exercise correlated positively with muscle performance (H2: $\rho = 0.34$, $P = .02$; H4: $\rho = 0.30$; $P = .06$), but within each strain this correlation disappeared. Dystrophin expression in *mdx-Xist*$^{\Delta hs}$ mice of both exercise groups combined positively correlated with performance of the two-, but not with the four-limb hanging test (H2; $\rho = 0.46$, $P < .01$, H4; $\rho = 0.14$, $P = .33$, Supplementary Fig. 1A–B).

Since voluntary exercise beneficially affected skeletal muscle function in all strains, we questioned whether exercise would also impact skeletal muscle integrity and histopathology. Therefore, we measured CK levels (marker muscle integrity) in plasma collected prior to sacrifice at 18 months of age and assessed the amount of pathology in the quadriceps. CK levels were elevated in *mdx* compared to wild type mice (which had CK levels < 500 U/L) (Fig. 1F), whereas CK levels in *mdx-Xist*$^{\Delta hs}$ mice were normalized to wild type levels. Voluntary exercise did not have a significant effect on CK levels in any of the strains, neither did CK levels correlate with dystrophin levels of the quadriceps in the *mdx-Xist*$^{\Delta hs}$ mice ($\rho = 0.23$, $P = .11$). Histopathology of the quadriceps was assessed on H&E stained sections. The amount of pathological tissue (containing fibrosis, necrosis and inflammation) was quantified on whole muscle cross-sections. The quadriceps of *mdx* mice was the most severely affected and this was partly prevented by low amounts of dystrophin in *mdx-Xist*$^{\Delta hs}$ mice (*mdx*: 12.7%; *mdx-Xist*$^{\Delta hs}$: 8.7%), shown in Fig. 1G. Although muscle pathology was less severe in *mdx-Xist*$^{\Delta hs}$ mice, it was still significant compared to wild type mice (BL10-WT: 2.8%; *Xist*$^{\Delta hs}$-WT: 4.0%). Voluntary exercise had no effect on the amount of pathology in any of the strains. The levels of histopathology did not correlate to dystrophin levels in *mdx-Xist*$^{\Delta hs}$ mice ($\rho = -0.23$, $P = .10$, Supplementary Fig. 1C). Performance of the H4-test, but not the H2-test, negatively correlated with the amount of pathology of the quadriceps in *mdx* mice (H4: $\rho = -0.54$, $P < .05$).

In *mdx* mice, the diaphragm is the most severely affected muscle. We therefore assessed how low dystrophin levels and voluntary exercise affect pathology in this muscle. Hereto, expression of genes involved in fibrosis (*Col1a1* and *Lox*) and inflammation (*Cd68* and *Lgals3*) was assessed (Fig. 2A–B). For all genes, expression levels were significantly elevated in *mdx* compared to BL10-WT mice. Low dystrophin levels in *mdx-Xist*$^{\Delta hs}$ mice marginally lowered expression, reaching significance for *Lox*, but expression levels remained elevated compared to *Xist*$^{\Delta hs}$-WT mice. Voluntary running increased *Col1a1* expression in *mdx-Xist*$^{\Delta hs}$ mice, but did not affect expression for other genes in any of the strains.

These data indicate that voluntary running positively affects muscle function and pathology not only in dystrophin negative *mdx* mice, but also when low dystrophin levels are expressed.

3.2. Low amounts of dystrophin preserve cardiac function in mdx-Xist$^{\Delta hs}$ mice

After subjecting mice to skeletal muscle function tests, we assessed body mass and cardiac function at 18 months of age, while after sacrifice, dystrophin levels of the heart were assessed for the *mdx-Xist*$^{\Delta hs}$ mice (Fig. 3A–B). As previously observed [40], levels in the heart were lower compared to the quadriceps (heart 9.9% *vs* qua 19.8%), and did not differ between sedentary and voluntary mice. We observed no significant correlation in dystrophin levels between heart and quadriceps muscles within individuals ($\rho = 0.27$, $P = .06$).

Body mass was not equally distributed between the strains ($P < .01$) and was not affected by voluntary exercise (Fig. 3C). Mice on a *Xist*$^{\Delta hs}$ background had a significant higher body mass ($P < .01$) compared to mice on a BL10 background. Because of this genetic background effect, all cardiac volumes were normalized to body mass. Cardiac hypertrophy was only present in *mdx* mice, as the heart-to-body-mass ratio was increased compared to all other strains ($P < .01$), shown in Fig. 3D. Although exercise increased heart-to-body-mass ratio in all strains ($P = .01$), this only reached significance in *Xist*$^{\Delta hs}$ -WT mice.

Heart rate was not significantly different between the strains and exercise regime (Fig. 3E). Left ventricular ejection fraction (LV EF) was significantly lower in sedentary and voluntary exercised *mdx* mice compared to all other strains (Fig. 3F), while low amounts of dystrophin normalized LV EF to wild type values (*mdx*: 49.6%; BL10-WT: 63.3%; *mdx-Xist*$^{\Delta hs}$: 58.4%; *Xist*$^{\Delta hs}$-WT: 60.2%). Voluntary exercise had no effect on LV EF. Left ventricular dilation, observed as an increased end-systolic volume, was only present in *mdx* mice and was prevented by low amounts of dystrophin in *mdx-Xist*$^{\Delta hs}$ mice (Fig. 3G). Voluntary exercise had no effect on left ventricular dilatation in any of the strains. Left ventricular stroke volume (LV SV) was not affected in the

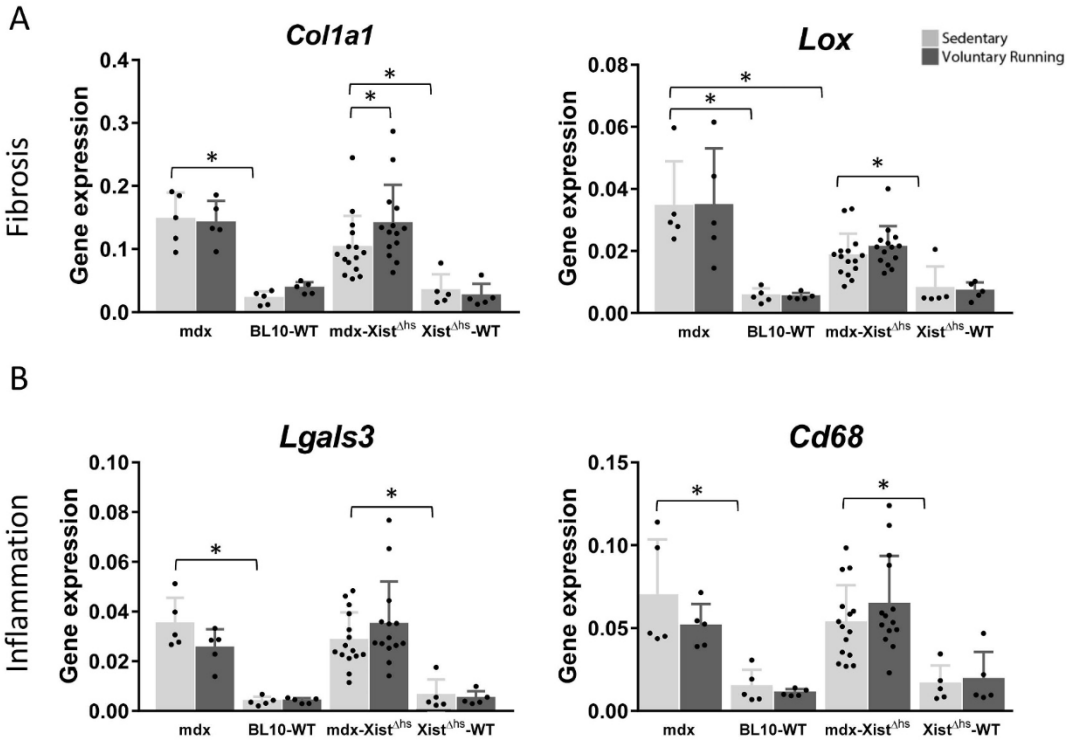


Fig. 2. Effect of low dystrophin levels and voluntary running on pathology of the diaphragm. Two-way ANOVA analyses and Fisher's least significant difference post-hoc tests were performed. Significant differences ($P < .05$) are indicated with the asterisks sign. Bar graphs represent the mean and error bars the standard deviation. A) Expression of genes involved in fibrosis (*Col1a1* and *Lox*) are elevated in *mdx* mice compared to wildtype. Low dystrophin levels partly normalize this towards wildtype levels reaching significance for *Lox*. Voluntary exercise only increased *Col1a1* levels in the *mdx-Xist*$^{\Delta hs}$ strain. B) Expression of genes involved in inflammation (*Cd68* and *Lgals3*) were increased in *mdx* and to a lesser extend in *mdx-Xist*$^{\Delta hs}$ mice. Voluntary running did not affect expression of inflammatory genes.

dystrophic strains (Fig. 3H). Although at strain level exercise increased LV SV ($P = .04$), it did not reach significance within each strain. A similar pattern was found for left ventricular cardiac output (LV CO), shown in Fig. 3I. Sedentary *mdx* mice had lower LV CO compared to all other strains but it was not significantly different compared to sedentary *mdx-Xist*$^{\Delta hs}$ or wild type mice (*mdx*: 0.53 *vs* BL10-WT: 0.62 *vs* *mdx-Xist*$^{\Delta hs}$: 0.62 *vs* *Xist*$^{\Delta hs}$-WT: 0.62 ml/min/g). Voluntary exercise increased LV CO at strain level ($P < .01$), but this only reached significance in BL10-WT mice (sed 0.62 *vs* vol run 0.78 ml/min/g, $P = .02$).

We also assessed functionality of the right ventricle. Sedentary *mdx* mice had significantly lower right ventricular ejection fraction (RV EF) compared to sedentary BL10-WT mice (*mdx* 42.3% *vs* BL10-WT 53.2%, $P = .03$), shown in Fig. 4A. Low amounts of dystrophin in sedentary *mdx-Xist*$^{\Delta hs}$ mice increased RV EF compared to *mdx* mice, but not up to wild type values (*mdx-Xist*$^{\Delta hs}$ 47.5% *vs* *Xist*$^{\Delta hs}$-WT: 56.8% $P < .01$). Voluntary exercise did not affect RV EF. The pronounced ventricular dilation found in the left ventricle was also present in the right ventricle (Fig. 4B). While sedentary *mdx* had significantly higher right ventricular end-systolic volumes (RV ESV) compared to sedentary BL10-WT mice (*mdx* 1.11 *vs* BL10 0.68 ml/g, $P = .01$), this was partly normalized in sedentary *mdx-Xist*$^{\Delta hs}$ mice (*mdx-Xist*$^{\Delta hs}$ 0.89 *vs* *Xist*$^{\Delta hs}$-WT 0.58 ml/g, $P = .01$). Voluntary exercise increased RV ESV in *mdx* mice. Right ventricular stroke volume and cardiac output did not differ between strains or groups (Fig. 4C + D).

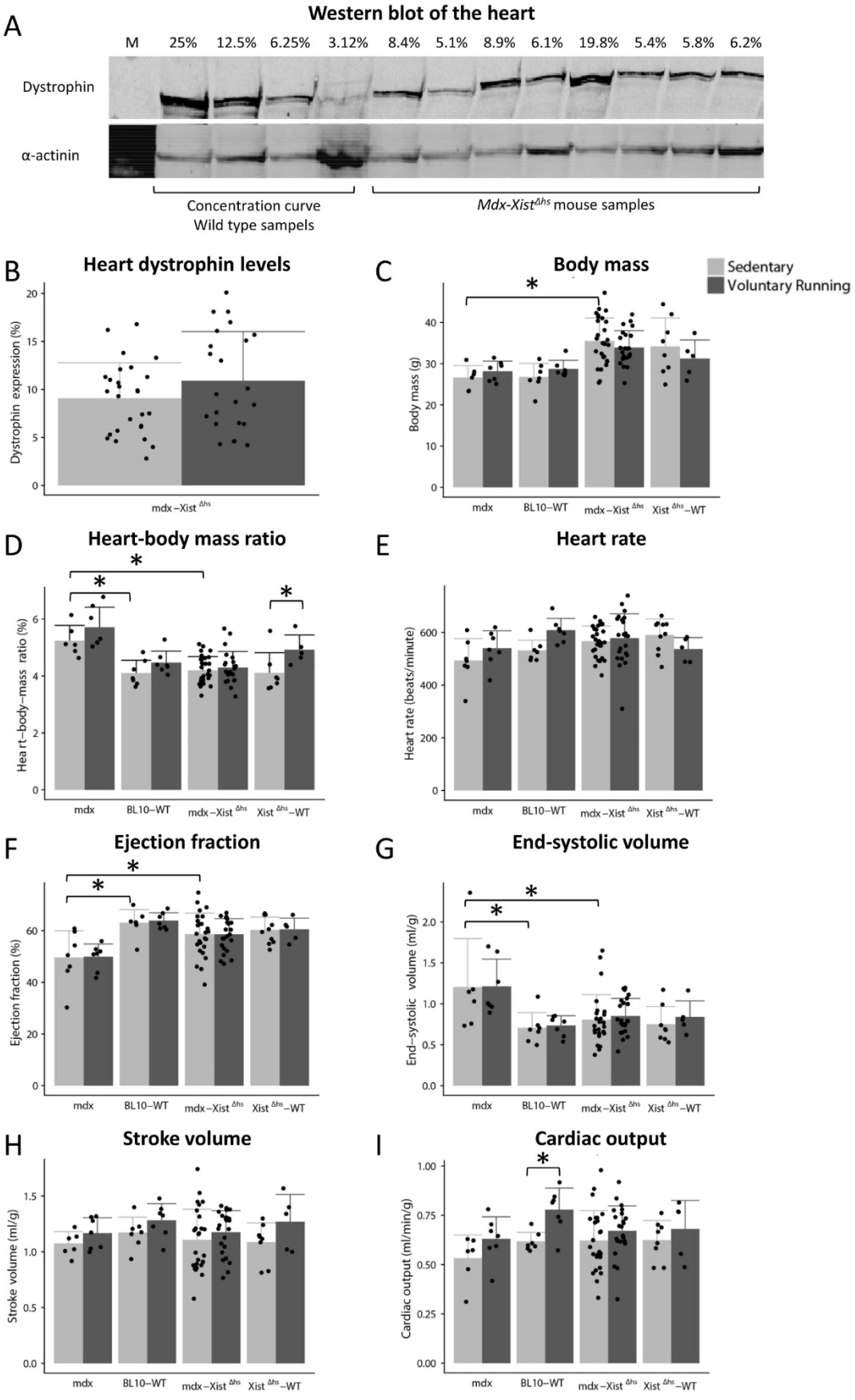
To determine if the improved cardiac function in *mdx-Xist*$^{\Delta hs}$ mice coincides with a decrease of heart pathology we assessed the amount of fibrotic infiltration on collagen type I stained sections (Fig. 5A). The most extensive amount of fibrosis was found in *mdx* mice (Fig. 5B). Sedentary *mdx* mice had significantly more fibrotic infiltration compared to sedentary *mdx-Xist*$^{\Delta hs}$ and wildtype mice (*mdx* 8.6% *vs* *mdx-Xist*$^{\Delta hs}$ 5.2%, $P = .04$; *mdx* 8.6% *vs* BL10-WT 2.2%, $P < .01$). Low dystrophin levels only partly prevented fibrotic infiltration, as fibrosis was still more extensive compared to wild type mice (*mdx-Xist*$^{\Delta hs}$ 5.2% *Xist*$^{\Delta hs}$-WT 1.1%, $P < .01$). Although cardiac function was improved by voluntary exercise, no significant effect on fibrosis was found.

Although low amounts of dystrophin partly prevented fibrotic infiltration, we observed no correlation between dystrophin levels and fibrosis in the hearts of *mdx-Xist*$^{\Delta hs}$ mice ($\rho = -0.20$, $P = .16$, Supplementary Fig. 1D). However, in *mdx* mice, the only strain that presented with cardiac hypertrophy, we observed a positive correlation between the amount of fibrosis and cardiac hypertrophy ($\rho = 0.67$, $P = .01$). This correlation was not found in other strains. In addition to this, the amount of fibrosis in *mdx* and *mdx-Xist*$^{\Delta hs}$ mice was significantly correlated to LV ejection fraction ($\rho = -0.43$, $P < .01$) and to LV ESV ($\rho = 0.41$, $P < .01$), but not to LV SV and LV CO. Furthermore, we observed a positive correlation in *mdx-Xist*$^{\Delta hs}$ mice between the amount of dystrophin in the heart and skeletal muscle function (H2: $\rho = 0.45$, $P = .00$; H4: $\rho = 0.46$, $P = .00$). In summary, these cardiac data reveal that voluntary running positively affects heart function, while it does not deteriorate heart pathology. Low dystrophin levels further increase this effect.

4. Discussion

In this study we investigated the effects of increased voluntary activity on skeletal and cardiac muscle function and pathology in mice expressing no or low dystrophin levels. We showed that although total distance run by *mdx* mice was less than wild type, this voluntary exercise did improve muscle performance in both strains. Notably, plasma CK levels and pathology of the quadriceps and diaphragm in *mdx* mice were not negatively affected by exercise. These results are in line with literature, where also others have reported on the beneficial effects of voluntary exercise in *mdx* mice [35–37].

Expression of low dystrophin levels in *mdx-Xist*$^{\Delta hs}$ mice improved the total distance run in the running wheel and more than doubled



(caption on next page)

Fig. 3. Cardiac dystrophin levels, body mass and left ventricular function assessed in 18-months-old mice. One and two-way ANOVA analyses and Fisher's least significant difference post-hoc tests were performed. Significant differences ($P < .05$) are indicated with the asterisks sign. Bar graphs represent the mean and error bars the standard deviation. A) Representative Western blot of the heart of *mdx-Xist*$^{\Delta hs}$ mice. To ensure equal loading in the concentration curve, wildtype protein lysates were diluted in *mdx* protein lysates. M = marker. B) Dystrophin levels were lower in the heart compared to the quadriceps muscle (Fig. 1A) and did not differ between sedentary or exercised mice. C) Body mass was lower in mice with a pure BL10 genetic background (*mdx* and BL10-WT) compared to mice with a Xist$^{\Delta hs}$ background, while exercise had no effect. D) In *mdx* mice a clear sign of cardiac hypertrophy was observed by means of the heart-to-body-mass ratio and this was not affected by exercise. Low amounts of dystrophin in *mdx-Xist*$^{\Delta hs}$ mice prevented cardiac hypertrophy. E) ANOVA analysis showed no differences in heart rate between the strains and exercise. F) Left ventricular ejection fraction was only impaired in *mdx* mice and not affected by exercise. G) Left ventricular dilatation was only present in *mdx* mice, prevented by low amounts of dystrophin in *mdx-Xist*$^{\Delta hs}$ mice and not affected by exercise. H + I) Left ventricular stroke volume and cardiac output were not different among strains and although ANOVA analysis revealed an increase in both cardiac parameters by exercise this only remained significant for cardiac output in BL10-WT mice.

hanging performance, although both did not reach significance. In addition to this, low dystrophin levels decreased plasma CK levels and partly prevented skeletal muscle and heart pathology. Dystrophin expression only correlated to muscle function (two limb hanging test), but not to pathology. This implies that voluntary exercise and low dystrophin levels are beneficial for skeletal muscle function and pathology in dystrophic mice.

The cardiac hypertrophy found in *mdx* mice is in accordance with other studies [24,46,47] and was slightly increased by voluntary exercise which is in line with a previous study which reported an increased heart-to-body-mass ratio from 5.0 to 6.0 mg/g after a two week voluntary exercise regime in 8–10 week old mice [48]. Increased cardiac hypertrophy becomes apparent in older *mdx* mice as reported by van Erp et al. [26], since at younger age the skeletal muscle hypertrophy results in increased body mass and therefore possibly mask the cardiac hypertrophy measured with heart-to-body-mass ratios. Cardiac hypertrophy correlated in *mdx* mice to the amount of cardiac fibrosis, while it did not correlate to heart function.

Bostick et al. already showed that carrier *mdx* mice, which express 50% dystrophin in heart, do not have an increased heart-to-body-mass ratio [49] and Jearawiriyapaisarn et al. showed that *mdx* mice treated with peptide-conjugated phosphorodiamidate morpholino oligomer which resulted in the expression of partially functional dystrophin levels of around 30% in cardiac muscle partly prevented cardiac hypertrophy in 7-months-old mice [47]. Our results imply that even lower amounts of dystrophin in *mdx-Xist*$^{\Delta hs}$ hearts (2.8–20.1%), prevent cardiac hypertrophy, since dystrophin expression in heart and cardiac hypertrophy are not significantly correlated. However, it should be noted that in our model the low levels are expressed from the fetal stage, while in the Jearawiriyapaisarn et al. study dystrophin was restored in adult mice.

We and others [50–52] found dilatation of the left ventricle in *mdx* mice. Low amounts of dystrophin prevented dilatation in *mdx-Xist*$^{\Delta hs}$ mice, while no correlation was found between dystrophin expression and severity of the left ventricular dilatation ($\rho = 0.08$, $P = .58$) and the amount of pathology in cardiac muscle ($\rho = 0.14$, $P = .33$). The right ventricle was dilated as well in *mdx* mice and this was only partly prevented in *mdx-Xist*$^{\Delta hs}$ mice. We used MRI to assess cardiac function which has superior resolution and ability to quantify cardiac volumes compared with ultrasound, but is not able to measure any regurgitation (backward flow) of blood. Since DMD patients and corresponding mouse models are not associated with valve problems, it is likely that

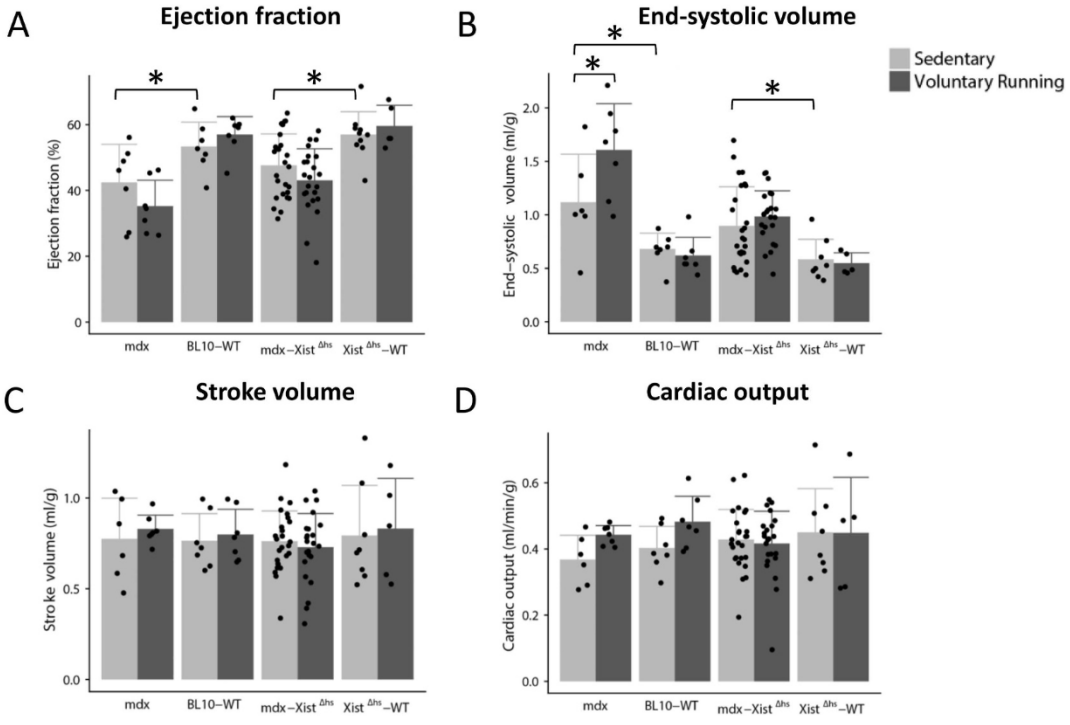


Fig. 4. Right ventricular function assessed in 18 months-old mice. Two-way ANOVA analyses and Fisher's least significant difference post-hoc tests were performed. Significant differences ($P < .05$) are indicated with the asterisks sign. Bar graphs represent the mean and error bars the standard deviation. A) Right ventricular ejection fraction in *mdx* was lower compared to BL10-WT mice. Low amounts of dystrophin increased ejection fraction in *mdx-Xist*$^{\Delta hs}$ mice, but not up to wild type levels. Voluntary exercise did not affect ejection fraction. B) Ventricular dilation by means of end-systolic volume was also present in the right ventricle of *mdx* mice and was partly normalized in *mdx-Xist*$^{\Delta hs}$ mice and only affected by exercise in *mdx* mice. C + D) Right ventricular stroke volume and cardiac output were not affected by strain or exercise.

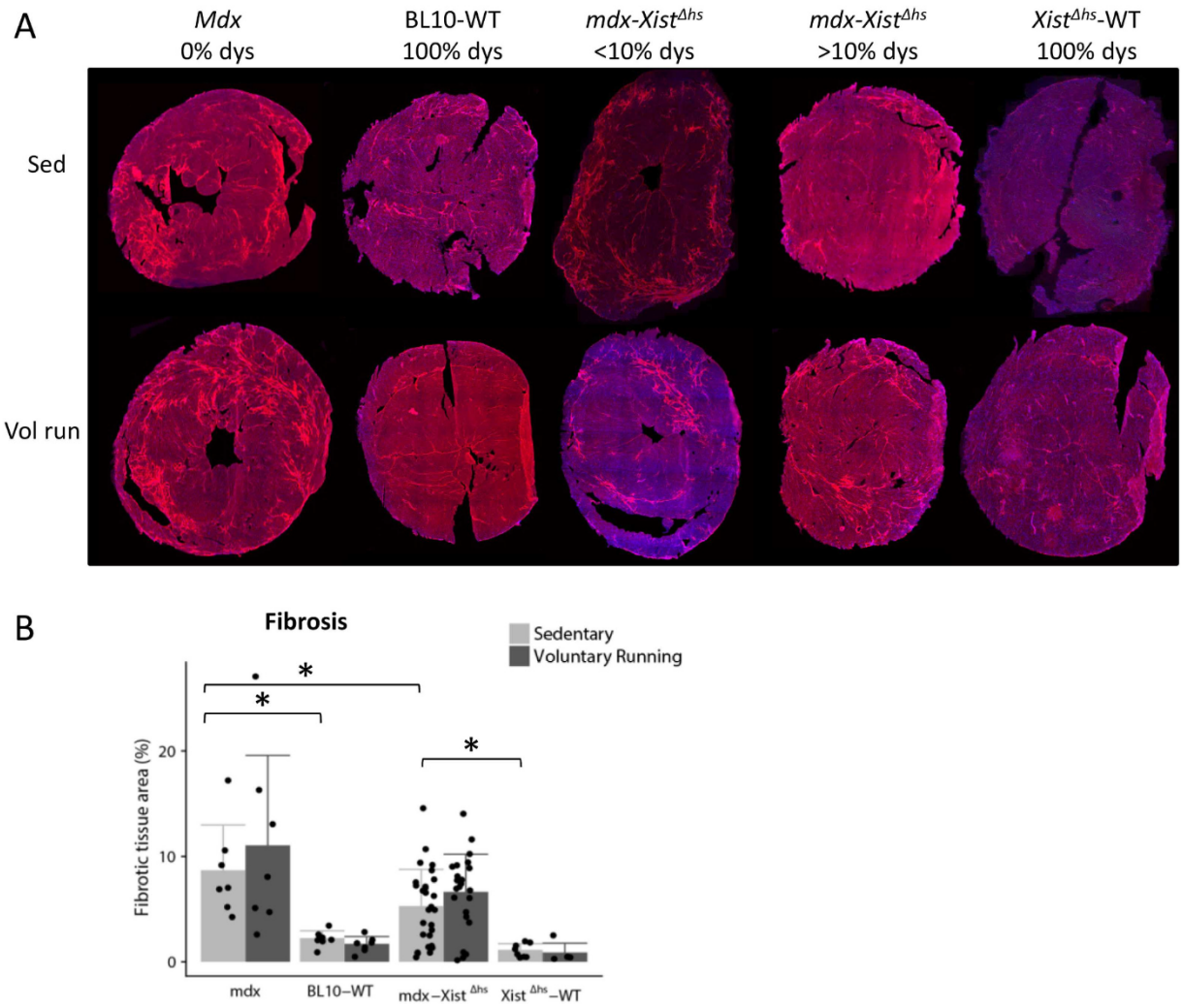


Fig. 5. Cardiac fibrosis in 18-months old mice. A) Representative pictures of the middle section of the heart stained for Collagen type I (red) and Dapi (blue). Extensive fibrosis can be observed in *mdx* mice. Sed = sedentary, Vol run = voluntary running group. B) Collagen staining showed most extensive fibrotic infiltration in *mdx* mice and this was partly prevented in *mdx-Xist*$^{\Delta hs}$ mice. Significant differences ($P < .05$) are indicated with the asterisks sign. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the cardiac output measurements are correct. Small discrepancies between the left and right ventricle functional parameters were present. These discrepancies could be attributed to difficulties in delineation of the endocardium at the base of the heart near the right atrioventricular valve, which cycles through the most basal plane of the MRI acquisition due to the cardiac cycle and has a thin free wall [55].

Based on previous studies, we assume that cardiac fibrosis was already present at the time of our voluntary exercise intervention in the *mdx* and the *mdx-Xist*$^{\Delta hs}$ models. It is therefore notable that voluntary exercise was not only beneficial for skeletal muscle function, it also resulted in increased cardiac function. In addition, expression of low dystrophin levels in *mdx-Xist*$^{\Delta hs}$ mice normalized cardiac output. Increases in heart rate and stroke volume contributed both to the increased cardiac output. This increase in cardiac function was not accompanied by worsening of cardiac pathology, as the amount of fibrosis, ejection fraction and severity of dilation was not changed. This implies that low intensity exercise can be safely performed, since no detrimental effect was found. Voluntary exercise was applied from an older age (15.5 months) onwards in this study. It would be interesting to assess the effects of such an intervention in younger mice on muscle function and pathology.

In the heart, we did not observe higher dystrophin levels in exercised mice compared to sedentary *mdx-Xist*$^{\Delta hs}$ mice, contrary to our observations in the quadriceps. Since we could only ascertain dystrophin levels after sacrifice at 18 months of age, randomization based on dystrophin levels prior to the experiment was not possible. It is possible that the higher dystrophin levels are due to chance. However, from wild type mice it is known that the pool of satellite cells, from which muscle cells can regenerate, expands by low intensity exercise [56] thereby improving regeneration capacity in the exercised mice. With a higher survival rate of dystrophin positive fibres over time, preferential differentiation and survival of dystrophin expressing muscle fibres could also lead to increased dystrophin expression. This is further supported by the equal dystrophin expression levels in hearts of sedentary and voluntary exercised *mdx-Xist*$^{\Delta hs}$ mice, since cardiomyocytes have a considerable lower regenerative capacity than skeletal muscle fibers [57]. We observed in voluntary exercised *mdx-Xist*$^{\Delta hs}$ mice higher skeletal muscle performance compared with the sedentary ones (doubling of hanging time in both hanging wire tests, though $P > .05$). This observation could also be explained by the hypothesized secondary effect of increased dystrophin levels caused by voluntary exercise. It should also be noted that *mdx-Xist*$^{\Delta hs}$ mice express low dystrophin levels from birth onwards, while dystrophin restoring therapies in DMD patients starts a later age. The effect of this restoration later in life is not known.

In summary we show that voluntary exercise is beneficial to skeletal

muscle and heart function in dystrophic mice while not affecting muscle pathology. Low amounts of dystrophin further improve skeletal muscle and cardiac function. These findings suggest that voluntary exercise may be beneficial for skeletal muscle and heart in DMD patients, especially in conjunction with low amounts of dystrophin.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yjmcc.2018.10.008.

Acknowledgements

We thank Ralf Werring for his help with post-mortem analyses and Ernst Suidgeest for assuring the quality of the cardiac contouring.

Author Contributions

MvP, LvdW, AAR and BK designed research; BK, KP, MH, CTdW and MvP performed research; BK and MvP analysed data; BK and MvP wrote the paper; BK, KP, MH, CTdW, LvdW, AAR and MvP reviewed and edited the paper.

Competing interest

The authors declare no competing interests.

Funding sources

This study was funded by a grant from the Duchenne Parent Project-NL to Annemieke Aarstma-Rus. Bauke Kogelman was supported by the European Union Bioimage-NMD project, Seventh Framework Programme (FP7/2007–2013) under grant 602485.

References

[1] A.E. Emery, The muscular dystrophies, Lancet 359 (2002) 687–695.
[2] L. Passamano, et al., Improvement of survival in Duchenne Muscular Dystrophy: retrospective analysis of 835 patients, Acta Myol. 31 (2012) 121–125.
[3] G. Nigro, L.I. Comi, L. Politano, R.J. Bain, The incidence and evolution of cardiomyopathy in Duchenne muscular dystrophy, Int. J. Cardiol. 26 (1990) 271–277.
[4] R.W. Kaspar, H.D. Allen, F. Montanaro, Current understanding and management of dilated cardiomyopathy in Duchenne and Becker muscular dystrophy, J. Am. Acad. Nurse Pract. 21 (2009) 241–249.
[5] D. D'Amario, et al., A current approach to heart failure in Duchenne muscular dystrophy, Heart 103 (2017) 1770–1779.
[6] M. Mehmood, et al., Comparison of right and left ventricular function and size in Duchenne muscular dystrophy, Eur. J. Radiol. 84 (2015) 1938–1942.
[7] G. Bosser, et al., Evidence of early impairments in both right and left ventricular inotropic reserves in children with Duchenne's muscular dystrophy, Am. J. Cardiol. 93 (2004) 724–727.
[8] P. Melacini, et al., Cardiac and respiratory involvement in advanced stage Duchenne muscular dystrophy, Neuromuscul. Disord. 6 (1996) 367–376.
[9] P. Melacini, et al., Myocardial involvement is very frequent among patients affected with subclinical Becker's muscular dystrophy, Circulation 94 (1996) 3168–3175.
[10] R.W. Kaspar, et al., Analysis of dystrophin deletion mutations predicts age of cardiomyopathy onset in Becker muscular dystrophy, Circ. Cardiovasc. Genet. 2 (2009) 544–551.
[11] D.M. Connuck, et al., Characteristics and outcomes of cardiomyopathy in children with Duchenne or Becker muscular dystrophy: a comparative study from the Pediatric Cardiomyopathy Registry, Am. Heart J. 155 (2008) 998–1005.
[12] E.M. Hoogerwaard, et al., Signs and symptoms of Duchenne muscular dystrophy and Becker muscular dystrophy among carriers in the Netherlands: a cohort study, Lancet 353 (1999) 2116–2119.
[13] L. Grain, et al., Cardiac abnormalities and skeletal muscle weakness in carriers of Duchenne and Becker muscular dystrophies and controls, Neuromuscul. Disord. 11 (2001) 186–191.
[14] R.S. Finkel, et al., Phase 2a study of ataluren-mediated dystrophin production in patients with nonsense mutation Duchenne muscular dystrophy, PLoS One 8 (2013) e81302.
[15] K. Bushby, et al., Ataluren treatment of patients with nonsense mutation dystrophinopathy, Muscle Nerve 50 (2014) 477–487.
[16] E.H. Niks, A. Aartsma-Rus, Exon skipping: a first in class strategy for Duchenne muscular dystrophy, Expert. Opin. Biol. Ther. 17 (2017) 225–236.
[17] Y.Y. Syed, Eteplirsen: first global approval, Drugs 76 (2016) 1699–1704.
[18] A. Aartsma-Rus, A.M. Krieg, FDA approves Eteplirsen for Duchenne muscular dystrophy: the next chapter in the Eteplirsen saga, Nucleic Acid Ther. 27 (2017) 1–3.
[19] C.A. Betts, et al., Implications for cardiac function following rescue of the dystrophic diaphragm in a mouse model of Duchenne muscular dystrophy, Sci. Rep. 5 (11632) (2015).
[20] S.M.G. Jirka, et al., Peptide conjugation of 2′-O-methyl phosphorothioate antisense oligonucleotides enhances cardiac uptake and exon skipping in mdx mice, Nucleic Acid Ther. 24 (2014) 25–36.
[21] H.M. Moulton, J.D. Moulton, Morpholinos and their peptide conjugates: therapeutic promise and challenge for Duchenne muscular dystrophy, Biochim. Biophys. Acta Biomembr. 1798 (2010) 2296–2303.
[22] S. Gielen, G. Schuler, V. Adams, Cardiovascular effects of exercise training: molecular mechanisms, Circulation 122 (2010) 1221–1238.
[23] G. Bulfield, W.G. Siller, P.A. Wight, K.J. Moore, X chromosome-linked muscular dystrophy (mdx) in the mouse, Proc. Natl. Acad. Sci. U. S. A. 81 (1984) 1189–1192.
[24] M. van Putten, et al., Low dystrophin levels in heart can delay heart failure in mdx mice, J. Mol. Cell. Cardiol. 69 (2014) 17–23.
[25] D.J. Stuckey, et al., In vivo MRI characterization of progressive cardiac dysfunction in the mdx mouse model of muscular dystrophy, PLoS One 7 (2012) e28569.
[26] C. Van Erp, D. Loch, N. Laws, A. Trebbin, A.J. Hoey, Timeline of cardiac dystrophy in 3-18-month-old MDX mice, Muscle Nerve 42 (2010) 504–513.
[27] J.T. Selsby, P. Acosta, M.M. Sleeper, E.R. Barton, H.L. Sweeney, Long-term wheel running compromises diaphragm function but improves cardiac and plantarflexor function in the mdx mouse, J. Appl. Physiol. 115 (2013) 660–666.
[28] A. Ferry, et al., Effect of voluntary physical activity initiated at age 7 months on skeletal hindlimb and cardiac muscle function in mdx mice of both genders, Muscle Nerve 52 (2015) 788–794.
[29] N.B. Wasala, B. Bostick, Y. Yue, D. Duan, Exclusive skeletal muscle correction does not modulate dystrophic heart disease in the aged mdx model of Duchenne cardiomyopathy, Hum. Mol. Genet. 22 (2013) 2634–2641.
[30] J.M. Costas, D.J. Nye, J.B. Henley, J.H. Plochocki, Voluntary exercise induces structural remodeling in the hearts of dystrophin-deficient mice, Muscle Nerve 42 (2010) 881–885.
[31] C. Hourdé, et al., Voluntary physical activity protects from susceptibility to skeletal muscle contraction-induced injury but worsens heart function in mdx mice, Am. J. Pathol. 182 (2013) 1509–1518.
[32] V. Brussee, F. Tardif, J.P. Tremblay, Muscle fibers of mdx mice are more vulnerable to exercise than those of normal mice, Neuromuscul. Disord. 7 (1997) 487–492.
[33] H. Radley-Crabb, et al., A single 30 min treadmill exercise session is suitable for 'proof-of concept studies' in adult mdx mice: a comparison of the early consequences of two different treadmill protocols, Neuromuscul. Disord. 22 (2012) 170–182.
[34] J.A. Call, J.N. McKeehen, S.A. Novotny, D.A. Lowe, Progressive resistance voluntary wheel running in the mdx mouse, Muscle Nerve 42 (2010) 871–880.
[35] K.A. Baltgalvis, et al., Exercise training improves plantar flexor muscle function in mdx mice, Med. Sci. Sports Exerc. 44 (2012) 1671–1679.
[36] J.A. Call, et al., Endurance capacity in maturing mdx mice is markedly enhanced by combined voluntary wheel running and green tea extract, J. Appl. Physiol. 105 (2008) 923–932.
[37] A. Hayes, D.A. Williams, Beneficial effects of voluntary wheel running on the properties of dystrophic mouse muscle, J. Appl. Physiol. 80 (1996) 670–679.
[38] A. Hayes, D.A. Williams, Contractile function and low-intensity exercise effects of old dystrophic (mdx) mice, Am. J. Phys. 274 (1998) C1138–C1144.
[39] A.E. Newall, et al., Primary non-random X inactivation associated with disruption of Xist promoter regulation, Hum. Mol. Genet. 10 (2001) 581–589.
[40] M. van Putten, et al., The effects of low levels of dystrophin on mouse muscle function and pathology, PLoS One 7 (2012) e31937.
[41] TREAT-NMD (Neuromuscular Network), Experimental protocols for DMD animal models, Available at: http://www.treat-nmd.eu/research/preclinical/dmd-sops/ (Accessed: 16th August 2017).
[42] R. van der Geest, P. de Koning, Mass. (The Division of Image Processing (LKEB)), Leiden Medical University Center, 2016.
[43] M. Hulsker, I. Verhaart, L. van Vliet, A. Aartsma-Rus, M. van Putten, Accurate dystrophin quantification in mouse tissue; identification of new and evaluation of existing methods, J. Neuromuscul. Dis. 3 (2016) 77–90.
[44] R Core Team, R: A Language and Environment for Statistical Computing, R Foundation for Statistical Computing, 2016.
[45] RStudio Team, RStudio: Integrated Development Environment for R, RStudio, Inc., 2016.
[46] B. Bostick, et al., AAV micro-dystrophin gene therapy alleviates stress-induced cardiac death but not myocardial fibrosis in > 21-m-old mdx mice, an end-stage model of Duchenne muscular dystrophy cardiomyopathy, J. Mol. Cell. Cardiol. 53 (2012) 217–222.
[47] N. Jearawiriyapaisarn, H.M. Moulton, P. Sazani, R. Kole, M.S. Willis, Long-term improvement in mdx cardiomyopathy after therapy with peptide-conjugated morpholino oligomers, Cardiovasc. Res. 85 (2010) 444–453.
[48] D.L. Allen, et al., Cardiac and skeletal muscle adaptations to voluntary wheel running in the mouse, J. Appl. Physiol. 90 (2001) 1900–1908.
[49] B. Bostick, Y. Yue, C. Long, D. Duan, Prevention of dystrophin-deficient cardiomyopathy in twenty-one-month-old carrier mice by mosaic dystrophin expression or complementary dystrophin/utrophin expression, Circ. Res. 102 (2008) 121–130.
[50] A. Crisp, et al., Diaphragm rescue alone prevents heart dysfunction in dystrophic mice, Hum. Mol. Genet. 20 (2011) 413–421.
[51] T.A. Meyers, D. Townsend, Early right ventricular fibrosis and reduction in biventricular cardiac reserve in the dystrophin-deficient mdx heart, Am. J. Physiol. Heart Circ. Physiol. 308 (2015) H303–H315.
[52] W. Zhang, et al., Abnormal cardiac morphology, function and energy metabolism in the dystrophic mdx mouse: an MRI and MRS study, J. Mol. Cell. Cardiol. 45 (2008) 754–760.
[55] F. Grothues, et al., Interstudy reproducibility of right ventricular volumes, function, and mass with cardiovascular magnetic resonance, Am. Heart J. 147 (2004) 218–223.
[56] G. Shefer, G. Rauner, P. Stuelsatz, D. Benayahu, Z. Yablonka-Reuveni, Moderate-intensity treadmill running promotes expansion of the satellite cell pool in young and old mice, FEBS J. 280 (2013) 4063–4073.
[57] M. Mercola, P. Ruiz-Lozano, M.D. Schneider, Cardiac muscle regeneration: lessons from development, Genes Dev. 25 (2011) 299–309.