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Low dystrophin levels in heart can delay heart failure in *mdx* mice

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ABSTRACT

Duchenne muscular dystrophy is caused by mutations that prevent synthesis of functional dystrophin. All patients develop dilated cardiomyopathy. Promising therapeutic approaches are underway that successfully restore dystrophin expression in skeletal muscle. However, their efficiency in the heart is limited. Improved quality and function of only skeletal muscle potentially accelerate the development of cardiomyopathy. Our study aimed to elucidate which dystrophin levels in the heart are required to prevent or delay cardiomyopathy in mice.

Heart function and pathology assessed with magnetic resonance imaging and histopathological analysis were compared between 2, 6 and 10-month-old female *mdx-Xist*$^{\Delta hs}$ mice, expressing low dystrophin levels (3–15%) in a mosaic manner based on skewed X-inactivation, dystrophin-negative *mdx* mice, and wild type mice of corresponding genetic backgrounds and gender.

With age *mdx* mice developed dilated cardiomyopathy and hypertrophy, whereas the onset of heart pathology was delayed and function improved in *mdx-Xist*$^{\Delta hs}$ mice. The ejection fraction, the most severely affected parameter for both ventricles, correlated to dystrophin expression and the percentage of fibrosis. Fibrosis was partly reduced from 9.8% in *mdx* to 5.4% in 10 month old *mdx-Xist*$^{\Delta hs}$ mice.

These data suggest that mosaic expression of 4–15% dystrophin in the heart is sufficient to delay the onset and ameliorate cardiomyopathy in mice.

1. Introduction

Duchenne muscular dystrophy (DMD) is the most common inherited neuromuscular disorder. In DMD patients, the synthesis of functional dystrophin proteins is prevented by mutations in the *DMD* gene [1]. Dystrophin negative fibers are vulnerable to exercise-induced damage, leading to loss of ambulation in the second decade of life. Cardiomyopathy is increasingly observed from 10 years of age onwards [2]. Old patients depend on assisted ventilation and at that stage the vast majority suffers from dilated and hypertrophic cardiomyopathy of the left ventricle, while the right ventricle and atrium remain unaffected [3,4]. Eventually, patients die of heart or respiratory failure in their third or fourth decade [5].

Becker muscular dystrophy (BMD) patients have in-frame mutations in the *DMD* gene, resulting in expression of internally deleted, but partly functional dystrophins. The skeletal muscle pathology is less severe than in DMD patients, but a more severe cardiomyopathy is observed, especially in BMD patients with a mild muscle phenotype, accounting for 50% of deaths [3,6,7]. Dilated cardiomyopathy is also occasionally found in DMD and BMD carriers, who express 50% of dystrophin in the heart [8].

There is no cure for DMD, but several potential therapies aiming at dystrophin restoration are under investigation in clinical trials [9–13]. Unfortunately, the efficiency of targeting the heart is limited. Eventually, this might result in partial dystrophin restoration in skeletal muscle, accompanied by improved muscle function, in the absence of restoration in the heart [14–16]. Given that BMD patients and some DMD/BMD carriers exhibit more pronounced cardiomyopathy than DMD patients, increased physical activity is likely to put a larger workload on the heart, thereby potentially exacerbating the development of cardiomyopathy, although other factors, like competition between utrophin and dystrophin, might also play a role.

The dystrophin-negative *mdx* mouse also suffers from dilated cardiomyopathy, of which the progression is accelerated by activity [17–21]. In line with this, partial dystrophin restoration in skeletal muscle improves voluntary activity, increasing the heart workload and exacerbating heart pathology [15]. By contrast, high dystrophin levels in skeletal muscles and diaphragm only, either improve heart function in 6 month old mice [22], or prevent worsening of heart failure in exercise challenged 23 month old *mdx* mice [23]. In addition, an internally deleted dystrophin (mini-dystrophin) transgene has been delivered to the heart using adeno

Abbreviations: DMD, Duchenne muscular dystrophy; BMD, Becker muscular dystrophy.

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associated viruses (AAV9). This improved heart function and partially prevented myocardial fibrosis, especially when mice were treated before they were 17 months [24,25].

From carrier *mdx* mice, it is known that 50% of dystrophin in the heart is sufficient to prevent development of heart pathology [26]. To study whether low dystrophin levels are enough to prevent or delay the onset of heart failure, we assessed heart function and pathology over time in *mdx-Xist*$^{\Delta hs}$ mice. These mice originate from crossing *Xist*$^{\Delta hs}$ females (expressing wild type dystrophin but carrying a mutation in the promoter of the *Xist* gene, which coordinates X-inactivation [27]), with *mdx* males (which do not express dystrophin and have a normal *Xist* gene). In the female offspring (*mdx-Xist*$^{\Delta hs}$), the X-chromosome expressing the mutated *Xist* gene, but intact dystrophin, is preferentially inactivated, resulting in expression of varying, low dystrophin levels in skeletal muscle and heart [28]. We show that mosaic expression of dystrophin in 4–15% of the cardiomyocytes (at wild type intensities) is sufficient to delay the onset of dilated cardiomyopathy and reduce the severity of pathology in *mdx* mice.

2. Methods

2.1. Animal care

Breeding pairs of *mdx* (C57BL/10ScSn-mdx/J) males and *Xist*$^{\Delta hs}$ females (BL/6 background) gave birth to *mdx-Xist*$^{\Delta hs}$ females [28]. The *Xist*$^{\Delta hs}$ model [27] was a kind gift from prof. Dr. Brockdorff (MRC Clinical Sciences Centre London, UK, current affiliation Department of Biochemistry, University of Oxford, UK). Genotyping was performed on DNA obtained from ear biopsies by PCR analysis. Mice were housed in individually ventilated cages with 12-h light-dark cycles and had *ad libitum* access to standard chow and water. All experiments were approved by the Animal Experimental Commission (DEC) of the LUMC and conform with the Directive 2010/63/EU of the European Parliament.

2.2. MRI heart function analysis

MRI analysis was performed at the Faculty of Science of the University of Leiden to which the mice were transported a week before analysis to acclimatize. Mice were anesthetized by inhalation of 2% isoflurane in a 1:1 mixture of pure oxygen and air. Respiration rate was monitored with a respiratory pad and kept constant at 50–80 respirations per minute. Mice were scanned in a vertical 9.4 T magnet with 89 mm bore size, equipped with a 1 T/m gradient system (Bruker BioSpin, Germany), using a quadrature birdcage coil (inner diameter 3 cm). Image acquisition was done with Bruker Paravision 5.0 software and took ~30 min including animal setup. A retrospectively-gated Intragate sequence was used with flip angle 10°; repetition time 8.5 ms; echo time 1.86 ms; field-of-view 2.56 × 2.56 cm^2; matrix 192 × 192; and in-plane resolution 133 μm. We made 8–9 short-axis slices of the heart of 1 mm thick and 200 repetitions per slice. The navigator echo was placed in-slice. Images were reconstructed to 18 frames per heart cycle. Per time point 27 *mdx-Xist*$^{\Delta hs}$ mice and six mice of the control groups were scanned. Six of the 135 mice scanned were not used for analysis as the angle of the scan was incorrect.

MASS for MICE software (developed at the LUMC, Division of Image Processing (LKEB)) was used for image analysis [29]. For each picture, endocardial and epicardial contours of both ventricles were drawn manually. The end diastolic and systolic phase were computed by the software and a maximum mass difference of 10% was accepted. When this criteria was met, end diastolic volume (EDV) and end systolic volume (ESV) were determined and based on these values stroke volume (SV = EDV-ESV), ejection fraction (EF = (SV/EDV)*100%) and cardiac output (CO = SV*heart rate) were calculated automatically. Left ventricular end diastolic thickness and end systolic wall thickening were measured for the mid-section of the heart.

2.3. Serum biomarker level analysis

For analysis of cardiac Troponin I and N-terminal Pro Brain Natriuretic Peptide (NT-proBNP) levels, blood (~1 ml) was drawn from the anesthetized mouse via the eye after which animals were sacrificed by cervical dislocation. Blood was collected in a non-coated eppendorf tube, allowed to clot at room temperature for 10 min and stored on ice. Samples were centrifuged at 1700 *g* for 10 min at 4 °C and stored at −80 °C. Levels of cardiac Troponin I and NT-proBNP were measured using the High Sensitive Mouse Cardiac Troponin-I ELISA Kit (Life Diagnostics, USA) and the ELISA Kit for NT-proBNP (Uscn Life Science Inc., China), respectively.

2.4. Histological examination

Tissues were snap frozen in 2-methylbutane (Sigma Aldrich, the Netherlands) cooled in liquid nitrogen. Sections of 8 μm were made on Superfrost Plus slides (Thermo Fishes Scientific, Menzel-Gläser, Germany) with a Shandon cryotome (Thermo Fisher Scientific Co., USA) along the entire length of the heart with an interval of 240 μm between the sections. Excess tissue was used for protein isolation. 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 DM RA2) was used to examine the sections at a 16 times magnification and images covering the entire heart were captured with a Leica DC350FX snapshot camera. The percentage of fibrotic tissue was assessed with ImageJ software (Rasband W.S., ImageJ, U.S. National Institutes of Health, USA, http://rsb.info.nih.gov/ij/, 1997–2008). The number of arterioles, cardiomyocyte size and distribution of fibrosis were determined on sections stained with Harris Haematoxylin and Eosin (Sigma Aldrich, the Netherlands), which were examined with a light microscope (Leica SP5, Leica Microsystems, United Kingdom). The minimal Feret's diameter of the cardiomyocytes was measured with ImageJ software on four pictures taken at a 40 times magnification. The severity of fibrosis, ranked into six categories, was assessed for ten regions to assess its distribution.

2.5. Dystrophin determination by western blot

The heart, diaphragm and quadriceps muscles were homogenized and dystrophin levels quantified by western blotting using the Odyssey system as described previously [28]. The lowest concentration in the standard curve was 3%. Blots were stained overnight with NCL-DYS1 (dilution 1:125, Novacastra, UK) and MF20 (MF20 dilution 1:20000, Developmental Studies Hybridoma Bank, University of Iowa, Iowa City) for dystrophin and myosin respectively. The fluorescent IRDye 800CW goat-anti-mouse IgG (dilution 1:5000, Li-Cor, USA) was used as secondary antibody.

2.6. Statistics

Statistical analyses were performed using SPSS 17.0.2. (SPSS, Inc., USA). Heart function was compared over time with the two-way analysis of variance (ANOVA). In case of significance ($P < 0.05$), a Fisher's Least Significant Difference-procedure (LSD) was applied to distinguish between heart function of different strains and to analyze within a mouse strain whether there are differences in heart function over time (so between 2, 6 and 10 month old mice). For ESV data, a log transformation was performed to correct for inequality of the data. Correlations for the 10-month-old *mdx-Xist*$^{\Delta hs}$ mice were tested with the Spearman correlation test. $P < 0.05$ was considered significant. The one-way ANOVA was conducted for comparison of the histological and wall thickness data. In case of significance ($P < 0.05$), the Bonferroni post hoc test was performed. For Fig. 1B, data are presented as medians.

3. Results

3.1. Low dystrophin levels in the heart prevent the age-dependent decline of heart function

To determine whether low dystrophin levels can prevent or delay the onset of dilated cardiomyopathy, heart function was determined by MRI in female *mdx* (no dystrophin), *mdx-Xist*$^{\Delta hs}$ (low dystrophin), C57BL/10ScSnJ (wild type) and *Xist*$^{\Delta hs}$ (wild type) mice aged 2, 6 and 10 months. We chose a cross-sectional design to allow post-mortem histological validation. Dystrophin was expressed in a mosaic manner in the hearts of *mdx-Xist*$^{\Delta hs}$ mice, where cardiomyocytes were either dystrophin positive at wild type intensities or negative as previously reported [28] (Supplementary Figure A). Dystrophin levels in the heart of *mdx-Xist*$^{\Delta hs}$ mice varied between 3 and 15% and were similar for the different age groups (2 months; 3–13.8%, 6 months; 3–14.9%, 10 months; 3–12.1%) as assessed by western blot (Figs. 1A–B).

The heart function of the two wild type models was largely comparable, but differed slightly, probably due to differences in the genetic background. In 2 month old *mdx* mice ejection fraction (EF) is stronger than that of wild type mice. With age, function of both ventricles deteriorated, most evidently for the (EF) and stroke volume (SV), while it remained constant in both wild type models (Figs. 1C–D, Supplementary Table A), as reported previously [30]. Expression of 3–15% dystrophin in the heart prevented EF and SV to drop in *mdx-Xist*$^{\Delta hs}$ mice as levels remained stable over time and were higher than *mdx* mice from the age of 6 months onwards ($P < 0.05$). The EF and SV of *mdx-Xist*$^{\Delta hs}$ mice were similar to levels of the wild type stains at 6 months, and for the left ventricle only lower than 10-month-old *Xist*$^{\Delta hs}$ mice ($P < 0.05$). The end systolic volume (ESV) of the left ventricle was increased in 10-month-old *mdx* mice ($P < 0.01$), while levels of *mdx-Xist*$^{\Delta hs}$ mice remained similar to wild types. The end diastolic volume (EDV) and cardiac output (CO) of both ventricles and ESV of the right ventricle did not differ between the mouse models.

End systolic mass of both ventricles increased with age in all mice. However, the left ventricular mass of *mdx* mice was greater than that of all other mice regardless of their age ($P < 0.01$), indicative of hypertrophy of the heart. This was not observed for *mdx-Xist*$^{\Delta hs}$ mice. The minimal Feret's diameter of cardiomyocytes showed a similar pattern, and tended to be larger in 10-month-old *mdx* than C57BL/10ScSnJ mice ($P = 0.056$, Supplementary Table B). The left ventricular lateral free wall thickness in diastole was larger in *Xist*$^{\Delta hs}$ than the other models aged 10 months ($P < 0.05$ Supplementary Table B), while thickening in systole was larger in *Xist*$^{\Delta hs}$ compared to both dystrophic models ($P < 0.01$). For both parameters no difference was observed in the interventricular septum.

Heart rate increased in *mdx* mice with age ($P < 0.001$), but remained stable over time for the other models. Heart rate of 2-month-old *Xist*$^{\Delta hs}$ mice was higher than that of both *mdx* and *mdx-Xist*$^{\Delta hs}$ mice ($P < 0.01$). In old *mdx* mice, heart rate exceeded both *mdx-Xist*$^{\Delta hs}$ and *Xist*$^{\Delta hs}$ mice ($P < 0.01$).

Dystrophin levels of 10-month-old *mdx-Xist*$^{\Delta hs}$ mice correlated with the EF of the left ventricle ($R = 0.464$ $P = 0.019$), which was also the most severely affected parameter in *mdx* mice (Fig. 2A). As heart function has been described to normalize by dystrophin expression in the diaphragm [22], we also assessed this for 10-month-old *mdx-Xist*$^{\Delta hs}$ mice. Dystrophin levels of the diaphragm varied between 1.5 and 38% (*mean* = 11.5% *stdev* = 8.8) and did not correlate to dystrophin levels in the heart ($R = 0.04$ $P = 0.849$). Dystrophin levels in the diaphragm only correlated with the EDV of the right ventricle ($R = 0.492$ $P =$

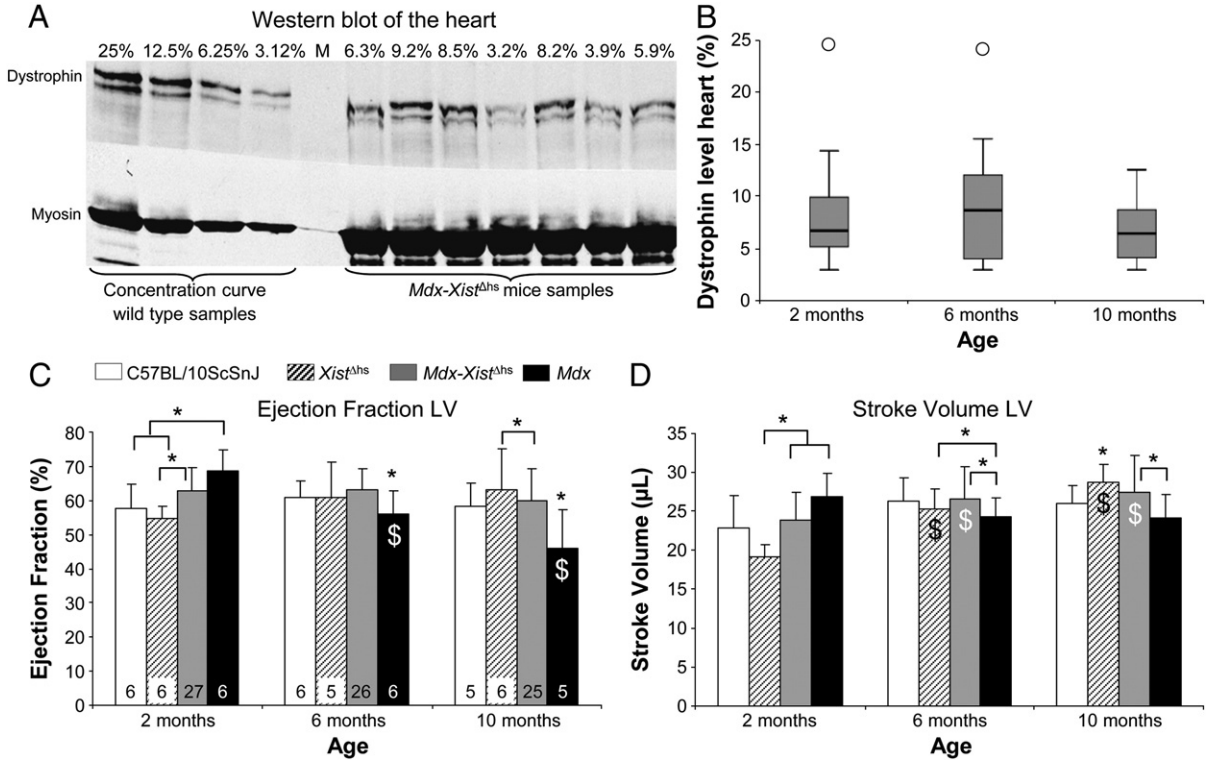

Fig. 1. Dystrophin levels and heart function of *mdx-Xist*$^{\Delta hs}$ mice. (A) Dystrophin levels were assessed by western blot for all *mdx-Xist*$^{\Delta hs}$ mice, as shown in the representative blot. Myosin was used as a loading control. M = marker. (B) Dystrophin levels plotted in the box plot. The line dissecting the box resembles the median and outer bars resemble the lower and upper quartile. The bars show maximum and minimum values considered excepting outliers (indicated by ○). Dystrophin levels varied between 3 and 15%, $n = 27$ for all age groups, and no increment was observed in older animals. (C) The EF of the left ventricle remained stable in wild type mice, but declined ($P < 0.01$) in *mdx* mice from the age of 6 months onwards, which was prevented in *mdx-Xist*$^{\Delta hs}$ mice. (D) The SV of the left ventricle was lower in old *mdx* mice than of *Xist*$^{\Delta hs}$ and *mdx-Xist*$^{\Delta hs}$. Bars represent mean values and the standard deviation. Asterisks indicate a difference of $P < 0.05$. Asterisks without brackets indicate a difference between that group and all other groups. $ indicates difference within that strain over time. Sample sizes are indicated in the bars.

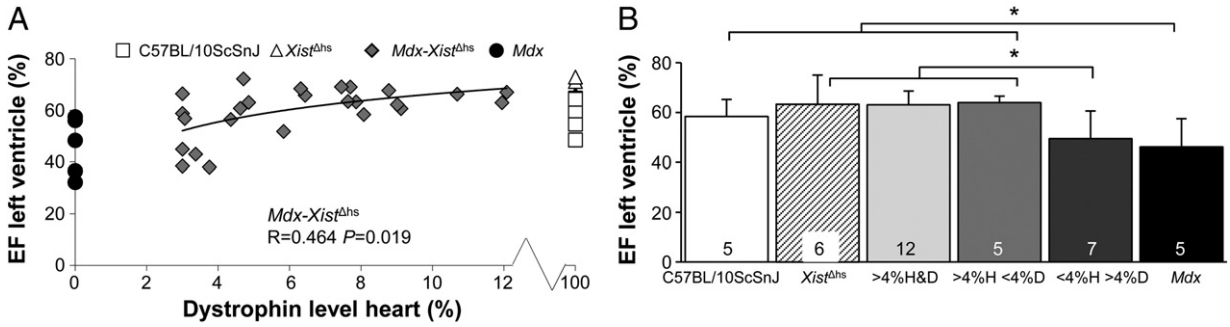

Fig. 2. Correlation between dystrophin expression and heart function. (A) Dystrophin levels of the heart correlated to EF of the left ventricle in 10-month-old *mdx-Xist*$^{\Delta hs}$ mice (R = 0.464 $P = 0.019$). (B) <4% dystrophin in the heart did not restore EF despite dystrophin expression in the diaphragm. EF levels were improved in mice expressing >4% dystrophin in the heart regardless of expression levels in the diaphragm. H = heart, D = diaphragm. Bars represent mean values and the standard deviation. Asterisks indicate a difference of $P < 0.05$. Sample sizes are indicated in the bars.

0.017), however, the EDV did not differ between any of the models. We hypothesized that the combined presence of dystrophin in the heart and diaphragm might have a cumulative beneficial effect on heart function (Fig. 2B). To study this, we grouped the EF of 10-month-old *mdx-Xist*$^{\Delta hs}$ mice based on their dystrophin levels in the heart and diaphragm into the following groups; mice with <4% dystrophin in the heart and >4% in diaphragm (heart; median 3.0% *stdev* = 0.29, diaphragm; median 8.67% *stdev* = 3.07), >4% dystrophin in the heart and <4% in diaphragm (heart; median 7.64% *stdev* = 1.13, diaphragm; median 2.63% *stdev* = 0.88) and >4% dystrophin in the heart and diaphragm (heart; median 6.31% *stdev* = 3.04, diaphragm; median 15.23% *stdev* = 9.31) (there were no mice with <4% dystrophin in both heart and diaphragm). The EF of both ventricles of mice expressing <4% dystrophin in heart and >4% in diaphragm was as low as age-matched *mdx* mice. The EF of mice expressing >4% dystrophin in heart and <4% or >4% in diaphragm was higher than in *mdx* mice ($P < 0.05$). This indicates that the expression of >4% dystrophin in the heart only improves EF of both ventricles, while expression of >4% (but less than 14%) dystrophin in diaphragm only does not (data for the right ventricle are not shown). We performed the same analysis for the quadriceps, but as for the diaphragm, no effect of dystrophin levels in quadriceps on heart function was found (data not shown).

We also assessed levels of potential serum biomarkers for heart failure; cardiac Troponin I and N-Terminal Pro Brain Natriuretic Peptide (NT-proBNP). Detectable concentrations of cardiac Troponin I were observed for some mice for all models and at all ages, but were slightly more often found in serum of dystrophic mice (Fig. 3A). Cardiac Troponin I levels did not differ between *mdx* and *mdx-Xist*$^{\Delta hs}$ mice, nor did they correlate to heart dystrophin levels (R = 0.004 $P = 0.986$). Levels did correlate to SV and CO of the right ventricle (R = −0.535 $P = 0.015$ and R = −0.483 $P = 0.031$ respectively). NT-proBNP levels were undetectable in any of the mouse models at any age.

3.2. Low dystrophin levels partly reduce fibrosis

Transverse cross-sections of the heart were stained with Collagen type I, to determine the percentage of fibrotic tissue (see Supplementary Figure B for representative examples). No fibrosis was detected in 2-month-old mice, whereas levels were slightly elevated in 6-month-old *mdx* and *mdx-Xist*$^{\Delta hs}$ compared to wild type mice (2.4% and 3.4% vs 2%, Fig. 3B). Due to high individual variation only the increment of the *mdx-Xist*$^{\Delta hs}$ mice reached significance ($P < 0.05$). Fibrosis was most prominent in 10-month-old *mdx* mice and was predominantly found in the lateral wall of the left ventricle (Fig. 3C). Its formation was partly prevented in *mdx-Xist*$^{\Delta hs}$ mice (9.8% vs 5.4%, $P < 0.01$), although levels were still higher than wild type ($P < 0.01$). Dystrophin levels in the *mdx-Xist*$^{\Delta hs}$ hearts did not correlate with fibrosis (R = −0.234 $P = 0.240$, Fig. 3D). Fibrosis negatively correlated to the EF of the left ventricle (R = −0.512 $P = 0.009$) (Fig. 3E). This concurs with the above described correlation between the EF and dystrophin levels. Heart mass of the left ventricle did not correlate with fibrosis (R = 0.253 $P = 0.222$). The number of arterioles did not differ between 10-month-old *mdx* and wild type mice (mean number of arterioles ±stdev *mdx* 4.75 ± 3.46 vs C57BL/10ScSnJ 4.25 ± 2.25, *Xist*$^{\Delta hs}$ 4.71 ±2.63, all groups $n = 6$).

4. Discussion

Potential therapeutic strategies for DMD are currently tested in clinical trials and encouraging results have been published for dystrophin restoration in skeletal muscles [12,13]. Unfortunately, several therapeutic approaches fail or are less efficient in restoring dystrophin expression in the heart [9–13]. It is unknown whether low dystrophin levels in the heart can preserve function and prevent or delay heart pathology.

We used skewed X-inactivation as a tool to generate mice expressing low dystrophin levels in a mosaic manner in the heart and skeletal muscles. Both C57BL/10ScSnJ and *Xist*$^{\Delta hs}$ (BL/6 background) wild type mice were investigated to eliminate potential strain effects. Heart function of *mdx* mice progressively declines with age, resulting in dilation of both ventricles in 10-month-old mice [18,19,30,31]. With age, the *mdx* heart becomes fibrotic, and genes involved in pathology are upregulated, whereas this remains stable in wild type hearts [32,33]. Interestingly, expression of 3–15% dystrophin in 10-month-old *mdx-Xist*$^{\Delta hs}$ mice ameliorates the age-dependent loss of heart function and pathology, as heart function parameters, fibrosis and hypertrophy partly normalize towards wild type levels. Although EF was higher in 2 month old *mdx* compared to wild type mice levels, they fell within the normal range. From the age of 6 months onwards, EF, SV and ESV are already affected in *mdx* mice, while this is observed later (10 months) and to a lesser extent in *mdx-Xist*$^{\Delta hs}$ mice. We did not observe a decrease in CO, probably due to an increased heart rate of the *mdx* mice, which was higher than all models except C57BL/10ScSnJ mice. This is likely due to the higher standard deviation in that group caused by two mice (st.dev. 80 bpm for C57BL/10ScSnJ while 40 bpm for the other models).

These data concur with a study we published recently involving a more severe mouse model also lacking utrophin (*mdx/utrn*$^{-/-}$*/Xist*$^{\Delta hs}$) [34]. Expression of 4% dystrophin in skeletal muscle greatly improved survival to over a year, whereas dystrophin and utrophin double knockout mice normally die before 12 weeks of age. Ejection fraction in 10 month old *mdx/utrn*$^{-/-}$*/Xist*$^{\Delta hs}$ mice with median heart dystrophin levels of 4.3% was severely reduced, but partly normalized in mice with median heart dystrophin levels of 29.7%. Here, we observe that EF partly normalizes to wild type levels in 10 month old *mdx-Xist*$^{\Delta hs}$ mice expressing lower dystrophin levels (<15%), indicating that utrophin-negative mice need higher dystrophin levels to improve cardiomyopathy to a similar extent. This results most likely from the

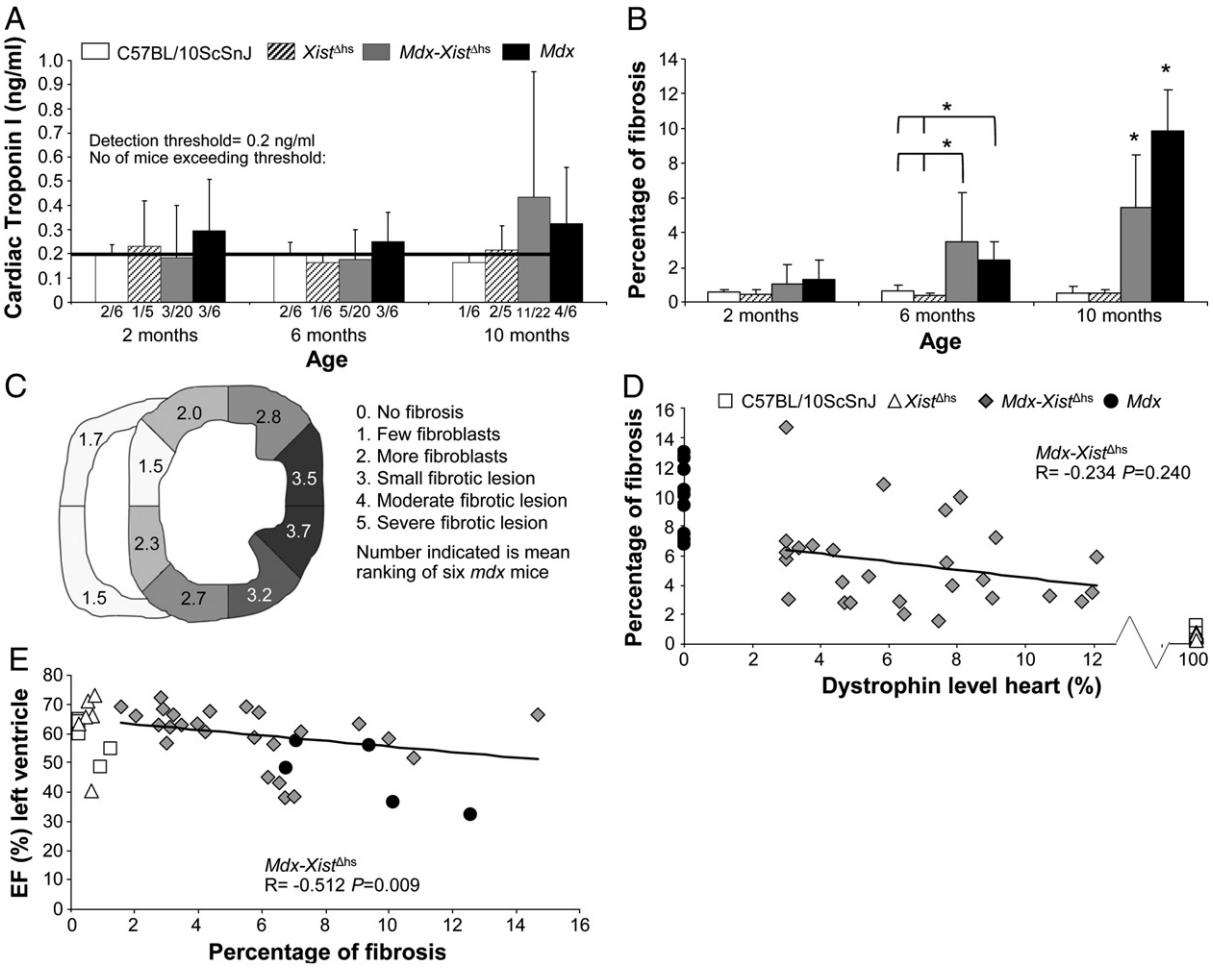

Fig. 3. Cardiac Troponin I levels, fibrosis and correlation between fibrosis, dystrophin expression and heart function. (A) Serum cardiac Troponin I levels exceeded the detection threshold of 0.2 ng/μl in some samples from all models. Levels did not differ between models or correlate to dystrophin expression in the heart ($R = 0.004$ $P = 0.986$). Sample sizes are indicated below the bars. (B) The percentage of fibrosis increases with age. While 10-month-old *mdx* mice develop severe fibrosis, low dystrophin levels partly prevent this. C57BL/10ScSnJ $n = 9$, *Xist*$^{\Delta hs}$ $n = 6$, *mdx-Xist*$^{\Delta hs}$ $n = 27$, *mdx* $n = 9$ per time point. (C) Distribution of interstitial collagen deposits of 10-month-old *mdx* mice based on ranking system. (D) Scatter plot of the percentage of fibrosis versus dystrophin levels of the heart of 10-month-old mice. Although *mdx-Xist*$^{\Delta hs}$ mice had less fibrosis than *mdx* mice, dystrophin levels did not correlate to fibrosis ($R = -0.234$ $P = 0.240$, *mdx* $n = 9$, C57BL/10ScSnJ $n = 9$, *Xist*$^{\Delta hs}$ $n = 6$, *mdx-Xist*$^{\Delta hs}$ $n = 27$). (E) Fibrosis levels of 10-month-old *mdx-Xist*$^{\Delta hs}$ mice negatively correlated to EF of the left ventricle ($R = -0.512$ $P = 0.009$). C57BL/10ScSnJ $n = 5$, *Xist*$^{\Delta hs}$ $n = 6$, *mdx-Xist*$^{\Delta hs}$ $n = 25$, *mdx* $n = 5$. Bars represent mean values and the standard deviation. Asterisks indicate $P < 0.05$. Asterisks without brackets indicate a difference between that group and all other groups.

protective effect of utrophin on the heart in *mdx* mice. Notably, there appears to be a threshold effect, where dystrophin levels <4% in the heart are not effective to ameliorate heart failure, while levels between 4 and 15% dystrophin are.

It was hypothesized that the combined expression of dystrophin in the heart and diaphragm might cumulatively improve heart function [22]. This was based on observations in Fiona mice and PPMO treated *mdx* mice expressing ~100% utrophin or dystrophin, respectively, in skeletal muscles. These high levels in diaphragm prevented heart failure in 6 month old mice [22]. High levels of expression of a human mini-dystrophin in diaphragm prevented worsening of heart function caused by increased activity in 23 month old *mdx* mice [23]. We observed that expression of 4–14% dystrophin in diaphragm does not improve heart function (EF, the most affected parameter) in *mdx-Xist*$^{\Delta hs}$ mice, whereas similar levels in the heart do. Expression of >4% dystrophin in both the heart and diaphragm does not further improve heart function indicating that the cumulative beneficial effect of dystrophin expression in diaphragm on heart function is limited. This is in line with observations in PPMO treated *mdx* mice, where increased voluntary activity deteriorated heart pathology, which could not be prevented by expression of 40–60% dystrophin in the diaphragm [15]. This indicates that this discrepancy might be caused by the low dystrophin levels in diaphragm in Malerba's and our study compared to the high dystrophin levels in the studies reported by Crisp [22] and Wasala [23]. This suggests that for a protective effect on heart function probably high dystrophin levels in diaphragm are needed.

Dystrophin levels of the *mdx-Xist*$^{\Delta hs}$ mice do not correlate with fibrosis, whereas, fibrosis correlates to the EF of both ventricles, which is in concordance with literature [33]. It should be stressed that *mdx-Xist*$^{\Delta hs}$ mice also express low dystrophin levels in skeletal muscle leading to improved muscle quality and function, which has been reported to increase the workload for the heart, thereby potentially worsening cardiomyopathy. We hypothesize that mice with dystrophin amounts in skeletal muscle that are sufficient to allow increased activity lead to a higher workload for the heart resulting in high levels of fibrosis, while mice with low dystrophin levels in the muscle and heart are less active, but still develop fibrosis because the lower dystrophin levels make the heart more sensitive even in an unstressed state. Whether 4–15% dystrophin in the heart is still protective when voluntary or forced strenuous exercise is applied or when the heart is stressed by drugs (e.g. dobutamine) remains to be elucidated.

It is good to bear in mind that, *mdx-Xist*$^{\Delta hs}$ mice express dystrophin from the embryonic stage onwards, whereas expression is normally restored at post weaning age in gene therapy treated *mdx* mice,

potentially giving the *mdx-Xist*$^{\Delta hs}$ mice an early start advantage. It is possible that the development of fibrosis is linked to ischemia, e.g. due to reduced capillary supply in the dystrophic heart. This was not assessed in the current study. However, it is known that dystrophin negative fibers are prone to damage and that the disposition and loss of nNOS are playing big roles in the development of heart failure, so ischemia may contribute to the pathology but is probably not the main driver.

The hearts of *mdx* mice are hypertrophic from the age of 2 months onwards, a pathological feature which is not observed in *mdx-Xist*$^{\Delta hs}$ mice. Our finding (assessing the mass of the entire ventricle) concurs with the report of Costas et al. (assessing ventricular wall thickness for a single point [20]), but is in contrast with work from others who observe hypertrophy from 6 months onwards (measuring heart to body weight ratios) [18,35]. The difference in age of onset might be caused by differences in sensitivity of the quantification methods used. Unfortunately, we did not collect data on heart and body weight, so it was not feasible to confirm this.

The applicability of cardiac Troponin I and NT-proBNP as biomarkers for heart failure was also assessed. Cardiac Troponin I has previously been used for detection of cardiac infarcts and was reported to be a useful marker for heart function in DMD and BMD patients [36]. Additionally, cardiac Troponin I levels of idebenone treated *mdx* mice have been reported to normalize towards wild type levels [37]. Unexpectedly, our serum cardiac Troponin I values did not correspond with those obtained by Buyse et al. However, personal communication revealed that these values were in the pg/ml range instead of the published ng/ml, making their observations fall in the same range. Although heart function of *mdx* mice is impaired, serum cardiac Troponin I levels barely exceed the detection threshold of the kit. Thus, we feel that the usefulness of cardiac Troponin I as a biomarker for cardiomyopathy in dystrophic mouse models is limited. Based on literature, the applicability of NT-proBNP is questionable as well, as positive and negative correlations with heart function in DMD and BMD patients have been published [4,38]. In our hands, NT-proBNP levels in serum are undetectable even in 10-month-old *mdx* mice. Therefore we propose that serum NT-proBNP levels are not suitable markers for the assessment of heart failure in *mdx* mice younger than 10 months.

Our study is partly limited by the fact that scans only entirely cover the left ventricle, while the top part of the right ventricle is not scanned. At initiation of this study, our primary focus was the left ventricle, but with interesting findings published on the right ventricle meanwhile, we also analyzed MRI data available for the right ventricle. All scans were analyzed in the same manner and up to the same height to ensure comparability. Lacking the upper ~25% of the right ventricle resulted in a lower CO for the right compared to the left ventricle. It may also be the reason that we observe a more severe phenotype for the left than the right ventricle, while others have reported the opposite [22,30].

Inherent to the *mdx-Xist*$^{\Delta hs}$ model, only females of a mixed background were assessed. Gender is known to influence cardiac function in animals and human. Female *mdx* mice aged 22 months develop more pronounced cardiomyopathy than males [39]. We can only speculate whether similar dystrophin levels will be protective in male mice as well. Since the *mdx* mouse has a BL/10 background and the *Xist*$^{\Delta hs}$ a BL/6 background, the resulting females have a mixed background. We therefore used two wild type models as controls, although we cannot rule out that heart function is impacted differently by loss of dystrophin in the two different backgrounds. An alternative strategy would have been to use one of the *mdx*^{cv} models, as these are in a BL/6 background. However, these models are not widely used and information on the development of cardiomyopathy in these models has only very recently become available [40].

In summary, we have shown that expression of 4–15% dystrophin not only delays the onset of cardiomyopathy, but also ameliorates its severity in 10-month-old *mdx* mice. These observations suggest that treatments restoring only low dystrophin levels in the heart of DMD patients may have benefits on heart function.

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Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2014.01.009.

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Glossary

Mdx: C57BL/10ScSn-Dmd^{mdx}/J, dystrophin deficient mouse