Long-term blinded placebo-controlled study of SNT-MC17/idebenone in the dystrophin deficient \textit{mdx} mouse: cardiac protection and improved exercise performance

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Aims
Duchenne muscular dystrophy (DMD) is a severe and still incurable disease, with heart failure as a leading cause of death. The identification of a disease-modifying therapy may require early-initiated and long-term administration, but such type of therapeutic trial is not evident in humans. We have performed such a trial of SNT-MC17/idebenone in the \textit{mdx} mouse model of DMD, based on the drug’s potential to improve mitochondrial respiratory chain function and reduce oxidative stress.

Methods and results
In this study, 200 mg/kg bodyweight of either SNT-MC17/idebenone or placebo was given from age 4 weeks until 10 months in \textit{mdx} and wild-type mice. All evaluators were blinded to mouse type and treatment groups. Idebenone treatment significantly corrected cardiac diastolic dysfunction and prevented mortality from cardiac pump failure induced by dobutamine stress testing \textit{in vivo}, significantly reduced cardiac inflammation and fibrosis, and significantly improved voluntary running performance in \textit{mdx} mice.

Conclusion
We have identified a novel potential therapeutic strategy for human DMD, as SNT-MC17/idebenone was cardioprotective and improved exercise performance in the dystrophin-deficient \textit{mdx} mouse. Our data also illustrate that the \textit{mdx} mouse provides unique opportunities for long-term controlled prehuman therapeutic studies.

Keywords
Muscular dystrophy • Therapy • Cardiomyopathy • Hemodynamics • Heart failure • Animal model

Introduction
Duchenne muscular dystrophy (DMD) is the most common and devastating type of muscular dystrophy worldwide, affecting one in 3500 live male births.\textsuperscript{1} This progressive and lethal X-linked myopathy is characterized by deficiency of dystrophin, a subsarcolemmal protein critical in membrane stabilization and prevention of contraction-induced cell membrane damage. Progressive striated muscle weakness and cardiomyopathy lead to severe disability and mortality of patients in their late teens to early twenties.

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Following implementation of ventilatory assistance to treat respiratory failure, heart failure has become a leading cause of death in DMD.1,3 In young DMD patients with still normal cardiac ventricular function at rest, reduced left ventricular contractile reserve (determined by inotropic stimulation with dobutamine) predicts later decline in cardiac function with age.4

Causative gene identification and pathophysiological insights have fuelled increasing therapeutic research efforts for DMD, of which the vast majority has been focused on the skeletal muscle involvement. Nevertheless currently no effective treatment exists, only corticosteroids have proved of some benefit but their long-term use is hampered by significant adverse effects. A major lesson from performed human trials that aimed to identify a disease-modifying treatment for DMD seems to be their apparently inevitable methodological shortcomings. Indeed, it is logic that the identification of a disease-modifying compound may require early-initiated (i.e. before onset of pathology) and long-term administration, but such type of therapeutic trial is not evident in human patients. We have performed such a trial of idebenone in the homologous dystrophin-deficient mdx mouse model of DMD,5 based on the drug’s potential to improve mitochondrial respiratory chain function and cellular energy production, as well as its potency to reduce oxidative stress.6,7 It was anticipated that early-initiated and long-term idebenone-mediated blocking of these important downstream effectors of dystrophin-deficiency would result in a reduced disease state in treated mdx mice at old age. Other than facilitating presymptomatic initiation and veritable long-term administration of treatment, the mouse model allowed invasive in vivo haemodynamic studies for the assessment of cardiac contractility. Limitations of the study were the required use of anaesthesia (for cardiac assessments) and the multiple endpoint testing.

Methods

Animals

Male wild-type (C57BL/10ScSn) and dystrophin-deficient mdx (C57BL/10ScSn-Dmd<sup>mdx<sup>15</sup></sup>) mice obtained from Jackson Laboratories were maintained at RCC Laboratory Services (Füllinsdorf, Switzerland). Sedentary mice (for cardiac assessments at age 10 months) from one litter were housed together from weaning to the end of the study period. Mice used for voluntary wheel running assessments (exercised mice) were housed individually in wheel cages from weaning to the end of the study period. All mice were kept under artificial light from 5 am to 5 pm and in darkness from 5 pm to 5 am. Weaning to the end of the study period. All mice were kept under artificial light from 5 am to 5 pm and in darkness from 5 pm to 5 am throughout their life span. All procedures were performed in accordance with the Belgian and Swiss regulations and under the required licenses, approval of procedures was obtained from the KU Leuven committee on the use and care of animals.

Treatment protocol, treatment groups, and flow of animals

The molecule investigated in this study is idebenone: 2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone (research code: ‘SNT-MC17; International non-proprietary name (INN): ‘idebenone’). A dose of 200 mg/kg bodyweight of either idebenone (‘ide’; SNT-MC17 from Santhera Pharmaceuticals, Liestal) or vehicle (‘veh’; placebo) was given once daily by gavage from the age of 4 weeks until the age of 10 months in mdx ('mdx-ide' group, n = 18, ‘mdx-veh’ group, n = 14) and wild-type mice (‘wt-veh’ group, n = 10). For wheel running performance (exercised mice), similar groups were treated from age 3 weeks until 12 weeks (wt-veh, n = 8; mdx-veh, n = 10; mdx-ide, n = 17). Animals were randomized per cage to the treatment groups. Litters of breeding cages were randomized to placebo (vehicle) and idebenone in an alternate fashion. All animals were included in the study between 17 April 2005 and 29 May 2005 (42 days). Vehicle consisted of 0.5% carboxymethylcellulose sodium salt (CMC, Fluka, Buchs, Switzerland) in water. To ensure accurate dosing of animals, idebenone was administered by gavage rather than by food mix. Since it was only feasible to administer animals once a day, a dose of 200 mg/kg/day was chosen as it represented a dose similar to the highest exposed animals (mice) in a therapeutic study in frataxin-deficiency.8 During the course of the study one animal in the mdx-veh group died around 7 months and one animal in the wt-veh group had to be killed at 6 months due to a tumor-like swelling in the abdomen. From one animal in the mdx-ide group there were no serum-derived measurements (cardiac biomarkers) available at baseline as the serum sample was lost in process. For the cardiac haemodynamic data assessments at age 10 months, three experiments were excluded because of technical reasons (problems with catheter insertion; two animals from mdx-ide group, one animal from wt-veh group), and one experiment was excluded because of a technical complication (liver tear) with haemodynamic instability (one animal in mdx-veh group). All animals allocated to the wheel running experiment completed the experiment.

Echocardiography at age 10 months

Mice were anaesthetized by 2% isoflurane inhalation. After shaving their chest, the animals were positioned in a left decubitus position on a heating pad for body temperature maintenance. Animals were imaged using a Vivid7 Dimensions (GE Vingmed, Horten, Norway) equipped with a 96-element linear array transducer (13L) transmitting at 14 MHz. Standard gray scale M- and B-mode images were acquired using a parasternal long- and short-axis view. Grayscale measurements were made off-line on a dedicated workstation (EchoPac, GE Vingmed). End-diastolic and end-systolic wall thickness of the anteroseptal (IVS) and inferolateral (PW) wall segments were measured together with the end-diastolic and end-systolic left ventricular internal diameter on both the M- and B-mode acquisitions from which fractional shortening (FS) was derived. From the average of the M- and B-mode assessment of ventricular morphology, LV myocardial volume (LVV), LV end-diastolic (EDV) and end-systolic (ESV) volumes were calculated using a half ellipsoid model of the LV. In this model, the long-axis dimension of the individual heart was taken into account by measuring it on the long-axis images. From these volumes LV ejection fraction (EF) was calculated. Finally, anteroseptal and inferolateral wall thickening was calculated as the change in thickness relative to the end-diastolic thickness and expressed as a percentage. All data were collected and analysed by two independent observers blinded to mouse type and randomization. For all measurements, the average value of both observers was used for statistical analysis.

In vivo cardiac haemodynamic measurements at age 10 months

Following anaesthesia for echocardiography, mice were allowed to recover completely for at least 3 days prior to haemodynamic assessment of cardiac contractility. For this, anaesthetized mice (intraperitoneal urethane 1200 mg/kg and alfa-chloralose 50 mg/kg) were tracheotomized and mechanically ventilated (Minivent 845; Hugo Sachs/Harvard Apparatus, March-Hugstetten, Germany). Body temperature was monitored with a rectal probe and maintained at 37 C.
with a heating pad. A precalibrated four-electrode pressure-conductance catheter (1.4 Fr, SPR-839; Millar Instruments, Houston, TX) was inserted in the right carotid artery and advanced into the LV to measure instantaneous intraventricular pressure and volume (PV-loops). After stabilization, baseline haemodynamic data were recorded. Subsequently LV preload was decreased by transient inferior caval vein occlusion (with cotton swab) while PV-loops (occlusion loops) were recorded, allowing to derive load-independent (intrinsic) contractility parameters. Afterwards, dobutamine was infused through a right jugular vein catheter at incremental doses of 1, 3, 10, and 30 ng/g/min each time for 2 min until a stable heart rate plateau was reached. During progressive dobutamine exposure, PV loops were recorded (allowing determination of contractile reserve). Next the dobutamine dose was stepwise reduced from 30 to 10 to 3 and to 1 ng/g/min, and each time occlusion loops were recorded. The parallel conductance attributed to tissues surrounding the LV cavity was estimated by bolus injection of 1.5 μl of 30% NaCl into the jugular vein. Blood conductance was determined at the end of each experiment using three precalibrated cuvettes, a conductance-volume calibration line was constructed with these cuvet data. Analysis was performed from each experiment with correction for parallel volume, and data were expressed in absolute volumes (PVAN software, Millar Instruments). All data are the average of at least five measurements during the experiment, each measurement representing at least 10 successive loops. Only technically acceptable loops (no arrhythmia, stable baseline) were included in the analysis for each experiment, which was performed blinded to mouse type and treatment groups.

**Cardiac histology at age 10 months**

After cardiac catheterizations, the animals were sacrificed, the hearts removed and fixed in a 6% formalin solution. The ventricles were cut into four slices parallel to the basis of the heart, and embedded in paraffin. Four-micron-thick histological sections were prepared in a standard way and stained with H&E and with Masson’s trichrome for collagen. Sections were examined by light microscopy for the presence of inflammation and fibrosis, blinded to mouse type and treatment groups. The RV, and the septum, the anterior, lateral and posterior wall of the LV were assessed separately. The proportion of fibrosis was quantified by a conventional point counting method. For each area, a total of 250 points were counted in three random fields, and the results were expressed as the proportion (in %) of the points hitting fibrosis. The amount of inflammation was scored from 0 (absent) to 3 (most severe case in our series) in five random fields per area. An average value was also calculated.

**Striated muscle histology at age 10 months**

The fibre diameter variability (minimal Feret’s diameter variance coefficients) and the percentage of centralized nuclei in the diaphragm and the quadriceps muscle were analysed as previously described.

**Cardiac biomarkers**

Cardiac troponin I (cTnI) levels were analysed in blood serum using the High Sensitivity Mouse Cardiac troponin-I Elisa Kit (Life Diagnostics, West Chester, USA). Blood samples were serially taken at ages 1, 6, and 10 months (retro-orbital sinus/plexus sampling; sample volume ~150 μL).

**Computerized wheel running analysis**

Voluntary exercise performance was monitored in parallel groups of mice from the age of 29 days (after 1 day acclimation to the monitoring equipment) until age 12 weeks. Voluntary running activity was measured with a computerized wheel system essentially as described previously. Wheel revolution counts were recorded every 10 s. Since all mice run extensively during the night and showed only little and irregular daytime running, only night-time running was included into the analysis. Beside the total daily running distance, a subset of running events (10 s time windows) with a wheel revolution above a speed threshold equivalent to 1.75 km/h was analysed separately.

**Statistical analyses**

(i) Echocardiography and cardiac haemodynamic measurements: Analysis was performed using Statistica 7.1, with Kruskal–Wallis ANOVA and χ² for post-hoc analyses (including Bonferroni adjustments). For the comparison of haemodynamic parameters at baseline (non-stress) conditions that could predict mortality during dobutamine stress Mann–Whitney U tests were used. (ii) Cardiac histology: In order to take into account the correlated nature of the data a first-order Generalized Estimating Equation (GEE) approach was employed using independent and exchangeable working correlations matrices. Marginal binary and ordinal logistic regression models were considered for the analysis of fibrosis and inflammation, respectively. For the analysis of inflammation, different models for ordinal data were intended. However, only proportional odds models were possible to be fitted due to sparseness of the sample. In each of the fitted models, the effect of the group, area, and group × area interaction term were included. The evaluation of the effect of the factors in the model was performed by using the Score-test for type 3 GEE analysis. Bonferroni adjustments to the significance levels for pairwise comparisons were performed when significant differences were found with the Score-test. Analyses were performed using SAS (9.1 for Windows). The GEE models were fitted using the procedure PROC GENMOD. (iii) Wheel running data: Analysis of the wheel running data (daily running distance, high speed running distance) integrating the whole analysed time period was done with a two-way ANOVA using the ‘aov’ method in the S-Plus software and with adapted (Bonferroni adjustment) significance levels for pairwise comparisons.

**Results**

**In vivo cardiac haemodynamics at age 10 months: idebenone prevents diastolic dysfunction**

The results of the comparison of the haemodynamic variables (under baseline conditions) between the different treatment groups at age 10 months are shown in Table 1. The heart weight (corrected for tibial length) was significantly higher in both mdx groups compared with the wild-type group, implying cardiac hypertrophy in mdx mice that was not corrected by idebenone treatment. Heart rates were similar in placebo-treated wild-type and mdx groups, but were higher in the idebenone treated mdx group. End-diastolic pressure, a measure of heart failure, was significantly increased in the placebo-treated mdx group, and corrected to wild-type levels in the idebenone-treated mdx group. In idebenone-treated mdx mice isovolumetric relaxation during diastole (Tau) was significantly faster than in placebo-treated mdx mice, and comparable with wild-type levels. Other load-dependent and -independent contractility measures under baseline conditions showed no further significant differences between the different groups.
Table 1 Comparison of haemodynamic parameters (mean ± SD) for wild-type vehicle-treated, mdx vehicle-treated, and mdx idebenone-treated mice at age 10 months

| Parameter | WT-veh n = 8 | mdx-veh n = 12 | mdx-ide n = 16 | ANOVA P-value |
|-----------|--------------|----------------|---------------|---------------|
| HW/TL (mg/cm) | 72.2 ± 8.6 | 82.5 ± 8.8** | 83.5 ± 9.9* | 0.022 |
| Parameters in steady state | | | | |
| HR (b.p.m.) | 501 ± 37 | 483 ± 36 | 532 ± 48** | 0.015 |
| Pmax (mmHg) | 82.6 ± 7.5 | 80.6 ± 8.7 | 84.3 ± 11.8 | 0.621 |
| Pes (mmHg) | 73.7 ± 10.8 | 72.2 ± 9.5 | 77.7 ± 12.6 | 0.423 |
| Ped (mmHg) | 3.6 ± 1.4 | 5.4 ± 2.2* | 3.4 ± 1.3** | 0.013 |
| Vcd (µL) | 30.1 ± 11.4 | 32.0 ± 7.5 | 32.1 ± 11.0 | 0.887 |
| SV (µL) | 16.1 ± 5.8 | 15.8 ± 3.6 | 15.9 ± 6.1 | 0.990 |
| EF (%) | 53.2 ± 11.7 | 47.6 ± 9.8 | 48.1 ± 11.1 | 0.483 |
| CO (µL/min) | 8149 ± 3189 | 7599 ± 1703 | 8639 ± 3846 | 0.689 |
| SW (mmHg·µL) | 1096 ± 445 | 1021 ± 367 | 1159 ± 612 | 0.775 |
| Ea (mmHg/µL) | 5.6 ± 3.8 | 4.8 ± 1.0 | 5.3 ± 2.0 | 0.630 |
| dP/dtmax (mmHg/s) | 6349 ± 1005 | 5907 ± 1623 | 7466 ± 3099 | 0.209 |
| dP/dtmin (mmHg/s) | -6257 ± 777 | -5564 ± 1321 | -6696 ± 1692 | 0.128 |
| Tau (ms) | 6.3 ± 0.9 | 7.0 ± 1.0 | 6.0 ± 0.9*** | 0.032 |

Parameters obtained after temporary preload reduction

| Parameter | WT-veh | mdx-veh | mdx-ide | ANOVA P-value |
|-----------|--------|---------|---------|---------------|
| PAMP (mW/µL²) | 88 ± 50 | 62 ± 27 | 76 ± 41 | 0.325 |
| Ees (mmHg/µL) | 8.4 ± 0.9 | 8.0 ± 1.1 | 8.2 ± 1.2 | 0.721 |
| PRSW (mmHg) | 71.4 ± 8.3 | 70.1 ± 13.8 | 70.7 ± 14.9 | 0.978 |
| EDPPVR (mmHg/µL) | 0.30 ± 0.17 | 0.42 ± 0.20 | 0.35 ± 0.14 | 0.260 |
| dP/dt,EDV (mmHg·µL/s) | 303 ± 91 | 359 ± 203 | 296 ± 81 | 0.456 |
| PVA (mmHg·µL) | 1328 ± 472 | 1310 ± 584 | 1281 ± 478 | 0.975 |
| Efficiency (%) | 58.1 ± 10.3 | 55.2 ± 9.8 | 61.3 ± 10.7 | 0.310 |

*P < 0.05 vs. WT-veh.
**P < 0.01 vs. WT-veh.
***P < 0.005 vs. mdx-veh.

HW/TL indicates heart weight corrected for tibial length; HR, heart rate; Pmax, maximum pressure; Pes, end-systolic pressure; Ped, end-diastolic pressure; Vcd, end-diastolic volume; SV, stroke volume; EF, ejection fraction; CO, cardiac output; SW, stroke work; Ea, arterial elastance; PAMP, preload adjusted maximal power; Ees, end-systolic elastance; PRSW, preload recruitable stroke work; EDPPVR, end-diastolic pressure–volume relationship; PVA, pressure–volume area.

Idebenone prevents dobutamine-induced cardiac failure in 10 month old mdx mice

Following baseline haemodynamic assessments, a dobutamine stress test was applied to assess cardiac contractile reserve (detection of subclinical cardiac failure) in the different treatment groups. Upon progressive challenge with the β-adrenergic receptor agonist dobutamine (incremental doses of 1, 3, 10, and 30 ng/g/min), already at low dobutamine doses placebo-treated mdx mice showed a 58% (7/12) mortality rate due to acute cardiac decompensation (Figure 1; lethal dobutamine doses: 1 ng/g/min for n = 3, 10 ng/g/min for n = 3, and 30 ng/g/min for n = 1). This cardiac failure was manifested by abrupt left ventricular dilatation and decreasing LV systolic pressures, and was not preceded by arrhythmias, indicating a primary heart failure based on diminished contractility. Cardiac pump failure during the dobutamine stress test was significantly prevented in mdx mice that had been treated with idebenone (Figure 1; reduction of heart failure and mortality to 19%, P = 0.033).

Given the pronounced differences in cardiac contractile reserve and mortality rate between idebenone- and placebo-treated mdx mice, we wanted to find parameters at baseline (non-stress) conditions that could predict mortality during the dobutamine stress test. Differences in all measured haemodynamic variables were analysed for mice that survived the stress test (pooled surviving animals from WT-veh, mdx-veh, and mdx-ide groups) vs. placebo-treated mdx mice that did not survive the stress test (Table 2). Non-surviving mdx-veh mice had a higher heart weight (cardiac hypertrophy) and showed reduced diastolic and systolic contractility at baseline conditions. Reduced values for the load-independent parameters PAMP (preload adjusted maximal power), Ees (end-systolic elastance), and PRSW (preload recruitable stroke work) in non-surviving mice indicate that it is a reduced intrinsic myocardial contractility that predicts lethal cardiac failure during dobutamine challenge.

Echocardiographic findings at age 10 months

The cardiac status of mdx and wild-type mice that had been long-term treated with placebo or idebenone was also evaluated by murine echocardiography at age 10 months (data summarized in Table 3). Morphological assessments showed significant differences (ANOVA) for end-diastolic diameters for the mdx mice. In mdx...
Table 2 Reduced diastolic and systolic contractility at baseline conditions (prior to inotropic challenge) predicts cardiac failure during dobutamine stress: comparison of baseline haemodynamic parameters (mean ± SD) of survivors (from all treatment groups) and non-survivors (from mdx-veh group only) of a physiological dobutamine stress test

|                          | Survivors of full protocol, $n = 25$ | Non-survivors (mdx-veh), $n = 7$ | P-value |
|--------------------------|--------------------------------------|---------------------------------|---------|
| HW/TL (mg/cm)           | 78.0 ± 9.0                           | 86.7 ± 9.4                     | 0.032   |
| Pmax (mmHg)             | 85.3 ± 9.6                           | 79.1 ± 9.3                     | 0.139   |
| Pes (mmHg)              | 77.6 ± 11.1                          | 71.9 ± 9.0                     | 0.225   |
| Ves (mL)                | 17.0 ± 6.5                           | 23.9 ± 6.0                     | 0.019   |
| EF (%)                  | 51.5 ± 8.3                           | 41.5 ± 4.3                     | 0.004   |
| dP/dtmax (mmHg/s)       | 7249 ± 2487                          | 5620 ± 1777                    | 0.117   |
| dP/dtmin (mmHg/s)       | 6658 ± 1326                          | 5302 ± 1560                    | 0.028   |
| PAMP (mW/μL)            | 85.8 ± 39.4                          | 45.1 ± 13.5                    | 0.013   |
| Ees (mmHg/μL)           | 8.46 ± 1.00                          | 7.72 ± 1.03                    | 0.094   |
| PRSW (mmHg)             | 73.6 ± 11.6                          | 64.0 ± 12.3                    | 0.068   |
| Tau (ms)                | 6.3 ± 0.9                            | 7.0 ± 1.0                      | 0.062   |

Histological studies at age 10 months: idebenone reduces cardiac inflammation and fibrosis

Results of morphometric analysis of cardiac inflammation and fibrosis in 10-month-old wild-type and mdx mice (placebo or idebenone treated) are shown in Tables 4 and 5. For fibrosis (Table 4), GEE logistic regression analysis showed that the odds of developing fibrosis was, respectively, 15.80 (adjusted CI: 4.89–49.46, $P < 0.0001$) and 11.36 (adjusted CI: 3.50–35.71, $P < 0.0001$) times higher in the mdx-vehicle and mdx-idebenone groups when compared with the wild-type-vehicle group. The odds of developing fibrosis was 1.39 times higher (adjusted CI: 1.07–1.85, $P = 0.0062$) in the mdx-vehicle compared with the mdx-idebenone group. The absence of a significant group x area interaction term in the models ($P = 0.2048$ and $P = 0.1999$ under the independent and exchangeable model, respectively) suggests that is not possible to reject the hypothesis of no difference in the probability of developing fibrosis among the groups across the heart areas. The wild-type-vehicle group showed no inflammation whatsoever (score 0 for all mice in all heart regions) (Table 5). Because of that, the wild-type-vehicle group was removed from the analysis. GEE regression analysis showed a non-significant group x area interaction suggesting that the differences in the distribution of inflammation scores between the two groups are the same across the heart areas. The results indicate that the percentage of subjects in high inflammation scores is significantly lower ($P = 0.0037$) in the mdx-idebenone compared with the mdx-vehicle group.

In skeletal muscle (quadriceps) and diaphragm, histological assessments of fibre diameter variability and percentage of fibres with centralized nuclei showed no significant differences between the mdx-idebenone group and the mdx-vehicle group, neither in 10-month-old sedentary mice, nor in 12-week-old exercised (voluntary wheel running) mice (no further data shown).

Effects of idebenone on biomarkers reflecting myocardial degeneration

Serum levels of cardiac Troponin I, a marker reflecting degree of active myocardial degeneration, were measured in the different treatment groups at different ages (at age 4 weeks prior to initiation of treatment, age 6 months, age 10 months) (Table 6).
Low levels were seen at age 4 weeks both in wild-type and mdx mice. Whereas cTnl levels remained low in wild-type mice at ages 6 and 10 months, at those ages levels were strongly elevated in mdx mice. Although at ages 6 and 10 months mean cTnl levels were lower in the mdx-idebenone compared with the mdx-vehicle group, indicating reduced myocardial degeneration, these differences were not statistically significant.

**Idebenone improves voluntary wheel running performance**

To evaluate if idebenone treatment improves the exercise performance of dystrophic mdx mice, voluntary wheel running performances of placebo (vehicle) treated wild-type mice and of placebo treated and idebenone treated mdx mice were recorded and analysed from the age of 29 days until age 12 weeks (Figure 2). Compared with vehicle-treated wild-type mice, daily running distances were significantly lower in the vehicle-treated mdx group. Idebenone treatment resulted in a significant and consistent improvement of daily running distances in mdx mice (Figure 2A). Cumulative running distance analysis revealed that idebenone treated mdx mice (mean 298 169 m) on average ran 57 507 metres more (95% CI: 1550–113 463, t-test: \( P = 0.045 \)) during the 58-day study period than vehicle-treated mdx mice (mean 241 184 m) (Figure 2B). Further analyses revealed that these differences in daily and cumulative running distances were not due to differences in daily running time (data not shown), but due to significantly improved high speed running with idebenone treatment resulting in a greater distance run above a pre-set threshold (1.75 km/h) for running speed (Figure 2C and D).
The main finding of our study is that presymptomatic-initiated and long-term idebenone treatment significantly corrected (prevented) cardiac diastolic dysfunction, blocked the development of lethal acute heart failure during a dobutamine-mediated stress protocol (improvement of contractile reserve), reduced cardiac inflammation and fibrosis, and improved voluntary running performance in the dystrophin-deficient mdx mouse. As such, this study provides the first evidence ever for a potential therapeutic role of idebenone in dystrophin deficient muscular dystrophy. Whereas mice are not man and future human studies will have to show whether therapeutic studies in a homologous phy. Whereas mice are not man and future human studies will have to show whether the improvements in exercise performance of these mice are linked to cardiac, skeletal muscle, and/or other changes.

The beneficial effects of idebenone can be explained by its ability to improve mitochondrial respiratory chain function and to reduce oxidative stress, pathways that have been implicated in the pathophysiology of dystrophin deficient muscular dystrophy. The absence of functional dystrophin protein causes sarcolemmal
instability and initiates a cascade of biochemical events in skeletal and cardiac muscle that ultimately leads to disintegration of muscle proteins and cell death. Impaired mitochondrial oxidative phosphorylation and increased formation of reactive oxygen species have been reported in \( \text{mdx} \) skeletal muscle, and oxidative damage has been reported to be involved in the pathogenesis of the heart failure that occurs in \( \text{mdx} \) mice.\(^{17-19}\) A recent study with \( \text{mdx} \) cardiomyocytes showed that excessive generation of reactive oxygen species is one of the key mechanisms that link the initial membrane fragility of the dystrophin-deficient cardiomyocyte to mitochondrial dysfunctions that precede cell death.\(^{20}\) Increased oxidative stress and damage has also been reported in human DMD patients, where interactions between the primary genetic defect and disruptions in the normal production of free radicals have been suggested to contribute to the pathogenesis of the disease.”

**Table 6** Cardiac biomarkers (cTnI; mean ± SD) in wild-type and \( \text{mdx} \) mice (vehicle or idebenone treated) at ages 1 month (prior to initiation of treatment), 6 months, and 10 months [cardiac Troponin I levels (ng/mL)]

| Age          | Group       | n  | WT-veh | mdx-veh | mdx-ide |
|--------------|-------------|----|--------|---------|---------|
| 1 month      | WT-veh      | 10 | 42 ± 15| 31 ± 7  | 31 ± 8  | 0.7     |
|              | mdx-veh     | 14 |        |         |         |         |
|              | mdx-ide     | 17 |        |         |         |         |
| 6 months     | WT-veh      | 9  | 42 ± 3 | 535 ± 177| 392 ± 146| 0.128   |
|              | mdx-veh     | 13 |        |         |         |         |
|              | mdx-ide     | 18 |        |         |         |         |
| 10 months    | WT-veh      | 9  | 61 ± 8 | 401 ± 239| 167 ± 28 | 0.254   |
|              | mdx-veh     | 13 |        |         |         |         |
|              | mdx-ide     | 18 |        |         |         |         |

**Figure 2** Idebenone significantly improves wheel running performance in \( \text{mdx} \) mice. Voluntary wheel running performance (mean ± standard error) in vehicle-treated wild-type, vehicle-treated \( \text{mdx} \), and idebenone-treated \( \text{mdx} \) mice. (A) Average daily running distance. (B) Cumulative daily running distance. (C) Speed of running. (D) Running distance at high speed. ANOVA and pairwise comparisons showed significant differences between all groups as well as between \( \text{mdx} \) vehicle and \( \text{mdx} \) idebenone groups (\( P < 0.001 \)) for the analyses presented in (A), (C), and (D). Data shown in (B) is a different graphical representation of the data shown in (A).
idebenone (2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone) has been shown to act as membrane-associated antioxidant that can prevent the formation of reactive oxygen species and reactive radicals.\(^{21–23}\) Idebenone inhibits lipid peroxidation which protects cell membranes and mitochondria from oxidative damage. The molecule’s optimized physicochemical properties favour uptake into cells and allow the incorporation into the mitochondrial membrane where it can facilitate the flux of electrons along the mitochondrial electron transport chain and increase cellular energy output which in turn protects mitochondria from malfunction and improves cellular survival.\(^{24}\)

In conclusion, presumptively-initiated and long-term treatment with idebenone was cardioprotective and improved running performance in the dystrophic-deficient mdx mouse. We have therefore identified a novel potential therapeutic strategy for the homologous human DMD, a currently untreatable disease where the associated cardiomyopathy is responsible for early death of \(\sim\)40% of patients. Our results therefore encourage studies with idebenone in human DMD patients. Such studies will also provide answers to the important question whether therapeutic studies in a homologous mouse model can be predictive for the human dystrophin-deficient situation. If so, our current study will gain further significance by demonstrating that the mdx mouse does provide unique opportunities for long-term controlled ‘prehuman’ therapeutic studies. Apart from its therapeutic findings, our study shows at a clinical level cardiac and motor deficits in the dystrophic mouse, and as such contributes to bridging the gap between laboratory scientists and clinicians in the field of muscular dystrophy.\(^{25}\)

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