



Mineralocorticoid receptor antagonism by finerenone is sufficient to improve function in preclinical muscular dystrophy

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Abstract

Aims Duchenne muscular dystrophy (DMD) is an X-linked inherited disease due to dystrophin deficiency causing skeletal and cardiac muscle dysfunction. Affected patients lose ambulation by age 12 and usually die in the second to third decades of life from cardiac and respiratory failure. Symptomatic treatment includes the use of anti-inflammatory corticosteroids, which are associated with side effects including weight gain, osteoporosis, and increased risk of cardiovascular disease. Novel treatment options include blockade of the renin–angiotensin–aldosterone system, because angiotensin as well as aldosterone contribute to persistent inflammation and fibrosis, and aldosterone blockade represents an efficacious anti-fibrotic approach in cardiac failure. Recent preclinical findings enabled successful clinical testing of a combination of steroidal mineralocorticoid receptor antagonists (MRAs) and angiotensin converting enzyme inhibitors in DMD boys. The efficacy of MRAs alone on dystrophic skeletal muscle and heart has not been investigated. Here, we tested efficacy of the novel non-steroidal MRA finerenone as a monotherapy in a preclinical DMD model.

Methods and results The dystrophin-deficient, utrophin haploinsufficient mouse model of DMD was treated with finerenone and compared with untreated dystrophic and wild-type controls. Grip strength, electrocardiography, cardiac magnetic resonance imaging, muscle force measurements, histological quantification, and gene expression studies were performed. Finerenone treatment alone resulted in significant improvements in clinically relevant functional parameters in both skeletal muscle and heart. Normalized grip strength in rested dystrophic mice treated with finerenone (40.3 ± 1.0 mN/g) was significantly higher ($P = 0.0182$) compared with untreated dystrophic mice (35.2 ± 1.5 mN/g). Fatigued finerenone-treated dystrophic mice showed an even greater relative improvement ($P = 0.0003$) in normalized grip strength (37.5 ± 1.1 mN/g) compared with untreated mice (29.7 ± 1.1 mN/g). Finerenone treatment also led to significantly lower ($P = 0.0075$) susceptibility to limb muscle damage characteristic of DMD measured during a contraction-induced injury protocol. Normalized limb muscle force after five lengthening contractions resulted in retention of $71 \pm 7\%$ of baseline force in finerenone-treated compared with only $51 \pm 4\%$ in untreated dystrophic mice. Finerenone treatment also prevented significant reductions in myocardial strain rate ($P = 0.0409$), the earliest sign of DMD cardiomyopathy. Moreover, treatment with finerenone led to very specific cardiac gene expression changes in clock genes that might modify cardiac pathophysiology in this DMD model.

Conclusions Finerenone administered as a monotherapy is disease modifying for both skeletal muscle and heart in a preclinical DMD model. These findings support further evaluation of finerenone in DMD clinical trials.

Keywords ACE/angiotensin receptors/renin–angiotensin system; Muscular dystrophy; Animal models of human disease; Cardiomyopathy; Mineralocorticoid receptors

Received: 16 June 2020; Revised: 29 July 2020; Accepted: 17 August 2020

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Introduction

Duchenne muscular dystrophy (DMD) is a genetic, progressive, neuromuscular disease caused by a mutation in the dystrophin gene and characterized by striated muscle degeneration.^{1,2} Subsequent conversion of muscle mass to fibrosis leads to loss of ambulation as well as cardiac and respiratory failure. About 1 in every 5000 boys is affected by this X-linked disorder,³ and there is still no long-term treatment.

Current standard-of-care treatment for DMD consists of corticosteroids beginning in early childhood, with additional nocturnal ventilatory support and angiotensin converting enzyme inhibitors (ACEis), respectively, as respiratory and cardiac function declines. Corticosteroids are used to treat inflammation and have immunosuppressant properties that temporarily extend ambulation and control symptoms in DMD patients.^{4,5} However, their use remains controversial, in part because the long-term corticosteroid dosing is associated with many negative side effects including weight gain, osteoporosis, hormone deficiencies, increased risk of cardiovascular disease, and adverse behavioural changes.^{6,7} Studies conducted in dystrophic mice also show continuous corticosteroid treatment leads to increased cardiac and muscle damage.^{8–10}

Negative consequences from long-term use of corticosteroids and the progressive cardiomyopathy present in DMD even with ACEi treatment^{11,12} justify the continued search for new therapies to improve the quality of life of DMD patients. Surprisingly, preclinical studies using DMD mouse models (*utrn*^{+/-}; *mdx* 'Het') demonstrated that treatment with mineralocorticoid receptor (MR) antagonists in combination with an ACEi not only improved cardiac function but also resulted in improved respiratory and limb muscle forces, reduction of ongoing muscle damage, and improved muscle membrane integrity.^{13–15} These studies have also demonstrated that non-specific MR antagonism (by spironolactone) and specific MR antagonism (by eplerenone) in respective combination with ACEi have comparable efficacy in muscular dystrophy in mice and that ACEi monotherapy improves muscle histopathology, but does not improve contractile function in DMD mice, strongly supporting an important role of MR in DMD pathophysiology.^{14,16}

MR are known to be present in many cell types including endothelial cells, myeloid cells and cardiomyocytes, and we showed that they are also present in all normal and dystrophic skeletal muscles.^{17,18} Pathophysiological conditions like elevated aldosterone release, high dietary salt load, or increased generation of reactive oxygen species can cause an MR overactivation with subsequent expression of pro-inflammatory and fibrotic proteins in the indicated cell

types, which ultimately lead to cardiovascular damage and dysfunction.¹⁹ Myeloid inflammatory cells are capable of synthesizing aldosterone and lead to increased aldosterone levels in dystrophic mouse muscles.²⁰ Blocking this signalling from chronic inflammation in dystrophic muscle likely explains the efficacy of MR antagonism.

Our team translated the preclinical cardiac benefits to a double-blind placebo controlled clinical trial with a 2 year extension study demonstrating that MR antagonism added to ACEi further prevents cardiac dysfunction in DMD patients compared with ACEi alone.^{21,22} We then demonstrated in a non-inferiority clinical trial equivalency between spironolactone and eplerenone on cardiac parameters in DMD patients.²³

Our recent studies have demonstrated that a conditional knockout of MR in myofibers reproduces many parameters of efficacy of ACEi + MR antagonism in a DMD mouse model, but *in vivo* functions of MR antagonism alone, without ACEi, have never been investigated.²⁴ An ongoing clinical study with spironolactone alone in young DMD boys warrants further preclinical investigation of the effect of MR antagonism as a monotherapy on the later onset cardiac dysfunction. Moreover, skeletal muscle gene expression changes have been shown to result from *in vivo* treatment of dystrophic mice with steroidal MR antagonists (MRAs) plus ACEi, but cardiac gene expression in dystrophic mice treated with MRAs alone are missing.^{13,17}

The steroidal MRA spironolactone binds MR at high affinity but has off-target effects on other hormone receptors including the androgen receptor, which causes the clinical side-effect gynaecomastia in post-pubescent males and influences treatment decisions in the male DMD population. While not shown in DMD trials to date, the steroidal MRAs spironolactone and eplerenone typically require careful monitoring for the potential adverse events of hyperkalaemia, particularly when given on top of inhibitors of the renin-angiotensin system such as ACEis or angiotensin receptor blockers to patients with concomitant kidney dysfunction.

Novel non-steroidal MRAs such as finerenone have been identified recently.^{25,26} These compounds have a different pharmacological profile in comparison with steroidal MRAs at least in preclinical studies.^{27,28} Finerenone has greater MR selectivity than spironolactone and higher receptor affinity than eplerenone *in vitro*.^{29,30} In a clinical trial of patients with chronic heart failure and chronic kidney disease, finerenone was at least as effective as spironolactone and was associated with significantly lower side effects.³¹ Preclinical studies demonstrated that there is a greater reduction in cardiovascular end-organ damage, fibrosis, and proteinuria with finerenone compared with eplerenone^{25,32,33} and

therefore generated the hypothesis that use of finerenone is associated with a more pronounced anti-hypertrophic/anti-fibrotic effect combined with a reduced risk of developing hyperkalaemia.^{34,35}

Therefore, it was the aim of the current study to investigate finerenone's disease-modifying efficacy as a monotherapy in a preclinical DMD model and to explore for the first time the cardiac expression profile in MRA-treated vs. untreated DMD animals.

Methods

Experimental animals and treatment

The Institutional Animal Care and Use Committee of The Ohio State University approved all protocols in compliance with the laws of the USA. All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All personnel performing experiments and collecting data were blinded to treatment and genotypes of the animals throughout the study.

Dystrophin-deficient, utrophin haploinsufficient (*utrn*^{+/-}; *mdx*) Het mice on a C57BL/10 background were bred in-house since their generation, genotyped as previously described, and used in this study.^{15,36} C57BL/10 mice bred in-house (originally obtained from Envigo) ($n = 18$; 11 male, 7 female) were used as wild-type controls. Het mice were randomly assigned into treated and untreated groups ($n = 18$ per group) keeping male and female groups equal (9 male and 9 female mice per group). All mice were housed in the same room under standard temperature, humidity, and light/dark cycle conditions. Het treated mice were given a custom diet Teklad Rodent Chow #7912 containing 100 ppm (parts per million) finerenone (Bayer AG, Wuppertal, Germany) prepared by Research Diets, Inc. Untreated Het mice and C57BL/10 wild-type control mice received Teklad Rodent Chow #7912 without medication. Medicated pellets were replaced every week. Body weights and food intake were monitored weekly to ensure an approximate drug dosage of approximately 3 mg/kg * day⁻¹. The finerenone chow dosage was calculated to reach a plasma concentration equivalent to once daily gavages between 1 and 10 mg/kg, which were previously shown to be efficacious in diverse chronic rat models.²⁵ A previous study in a mouse disease model revealed a free plasma drug concentration of 28 µg/L (equivalent to about 3xIC₅₀ of finerenone at MR *in vitro*) after 4 weeks on a custom diet with 100 ppm finerenone.³⁷ This plasma exposure is similar to steady-state exposures in male CD-1 mice after a once daily gavage of 3 mg/kg (Bayer AG).

Mice were started on treatment at 4 weeks-of-age at weaning or left untreated and were all sacrificed at

20 weeks-of-age. Mice were euthanized by cervical dislocation without anaesthesia following the institutional animal care and use committee and American Veterinary Medical Association guidelines.

In vivo grip strength measurements

A grip strength meter (Columbus Instruments) was used to evaluate forelimb muscle strength according to the methods reported previously.³⁸ In brief, prior to the initiation of the experiments, mice were trained during two sessions occurring at least 2 days apart. At least 2 days after the second training period, five pulls were recorded by allowing the mice to grasp the bar on the meter and then pulling them gently by the tail. Each group of five pulls was followed by a 1 min rest and this procedure was repeated five times. The highest value in the first trial was used as the peak force produced from rested mice, and the highest value in the fifth trial was used as the peak force produced in fatigued mice. Exclusion criteria were set prior to commencing the study and included any pull in which the mouse grasps with only one forepaw or without resistance. The absolute grip strength in Newtons (N) recorded automatically on a digital force transducer (Chatillon, #DFE2-002) and the weight of each mouse (g) was used to calculate grip strength force normalized to body weight. The same researcher, blinded to genotypes and treatment, performed all measurements in the vivarium where the animals were housed.

In vivo cardiac measurements

Magnetic resonance imaging (MRI) was performed on treated and untreated Het mice ($n = 12$ per group) 4 days before completing the treatment of the mice. During imaging, mice were anaesthetised with 1.5% isoflurane and electrodes for detection of the ECG signal were used, while animal temperature was maintained at 37°C in a 9.4 Tesla 30 mm bore system (Bruker Biospin) as previously described.¹⁵ Heart rate (HR) was maintained at a minimum of 350 bpm during the imaging. Myocardial strain and strain rate were recorded using vector-based tracking software (Vector Velocity Imaging, Siemens).

Electrocardiography (ECG) was performed *in vivo* in conscious and unrestrained mice just before euthanasia at 20 weeks-of-age. The body weight of each mouse was recorded, and ECG measurements were performed using the ECGenie system (Mouse Specifics, Inc.). Briefly, an ECG recording of approximately 8–10 s from each mouse was analysed during the time intervals when paws were in contact with the electrodes during a constant HR.¹⁶ ECG measurements included QT interval, HR, as well as QT adjusted for HR variability (QTc).

In vitro extensor digitorum longus and diaphragm contraction force

Contractile functions of *extensor digitorum longus* (EDL) muscle were assessed according to methods described previously.^{14,38} Briefly, isolated EDL muscle from each mouse was carefully dissected, and muscle was stretched to the optimal length while incubated at 30°C in Krebs–Henseleit solution continuously cycled. After 10 min, the muscles were subjected to tetanic contraction by stimulating for 250 ms at 150 Hz to measure baseline specific force. After a 5 min rest period, six eccentric contractions were performed by stimulating the muscle for 450 ms at 150 Hz with 3% stretch for the final 200 ms of the contraction. The first five stimulations were followed by a 5 min rest, and the fifth and sixth contraction were separated by a 15 min rest period. After the eccentric contraction, protocol was completed, muscles were weighed, and data were analysed using LabVIEW software (National Instruments, Austin, TX, USA). Force was normalized to cross-sectional area to determine specific force.

Diaphragm force measurement was assessed as we have previously reported.¹⁴ Diaphragm strips were carefully dissected (~3 mm wide). Platinum–iridium field electrodes were placed around strips at a controlled temperature of 37°C to stretch the muscle to the optimal length. After 10 min rest period, the muscle was stimulated for 250 ms during six tetanic contractions of 20, 50, 80, 120, 150, and 180 Hz with 2 min of rest between contractions. Output was recorded using LabVIEW (National Instruments), and specific force was expressed per unit cross sectional area (mN/mm²).

Histopathology analyses and quantification

The heart from each mouse in the study was divided transversely, and the top half was embedded in optimal-cutting temperature freezing medium on isopentane cooled in a bath of liquid nitrogen and then stored at –80°C. Quadriceps muscles were removed, cut transversely, and processed using the same method. Sections of 8 µm thickness were cut from each frozen muscle using a cryostat model OTF 5000 (Bright). Muscle sections were stained with haematoxylin and eosin to assess quality before any fluorescent antibody staining. For immunofluorescence staining, heart and quadriceps sections were incubated with an antibody against mouse IgG (Alexa 488 goat anti-mouse IgG, 1:200; Life Technologies) to quantify muscle damage. Heart muscle sections were also stained with rabbit anti-mouse fibronectin (1:40, Abcam, ref# ab23750) and incubated with Alexa 555 goat anti-rabbit IgG secondary antibody (1:200, Life Technologies, ref# A21429). Sections were mounted with VECTASHIELD mounting medium (H-1000, Vector Laboratories) and counterstained with DAPI. Immunofluorescence stains were photographed using a Nikon Eclipse 800 microscope under a 10× objective with

a SPOT RT slider digital camera and software, and images were processed with Adobe Photoshop CS6 software. Damage was quantified by the same individual who was blinded to genotype and treatment for all samples of the same tissue type and is reported as percentage of cross-sectional area, as previously described.^{14,16,38}

RNA isolation and microarray analysis

Liquid nitrogen frozen heart ventricular tissues from three biological replicates from finerenone-treated and untreated Het mice were pulverized with a mortar and pestle, and RNA was isolated with TRIzol reagent (Life Technologies #15596026), following the manufacturer's instructions. Only male mice were used for this experiment to limit variability. The RNA samples were DNase-treated using RQ1 DNase (Promega #M610) and further purified using RNeasy mini kit (Qiagen #74104) as previously described.^{13,39} RNA integrity was analysed with an Agilent 2100 Bioanalyzer (Agilent Technologies), and all samples had RNA integrity numbers above 7.3 and 260/280 ratios between 1.7 and 2.1.

One hundred nanograms of the RNA samples were lineally amplified, and 5.5 µg cDNA was labelled and fragmented using the GeneChip WT PLUS Reagent Kit (Affymetrix, Santa Clara, CA, USA), following the manufacturer's instructions. Labelled cDNA targets were hybridized to Affymetrix GeneChip® Clariom D array, mouse for 16 h at 45°C, rotating at 60 rpm. The arrays were washed and stained using the GeneChip Fluidics Station 450 and scanned using the GeneChip Scanner 3000 7G. Arrays were normalized using the gene-level signal space transformation robust multi-chip analysis algorithm in Expression Console software and comparisons were made in Transcriptome Analysis Console software (Affymetrix) using a cut-off of two-fold. Gene groups were assigned using the Functional Annotation clustering tool from the Database for Annotation, Visualization and Integrated Discovery (DAVID). Microarray data have been deposited in NCBI GEO under accession number GSE150302.

Data and statistical analysis

Summary values are presented as mean ± SEM and analysed using Minitab (Minitab, LLC, State College, PA, USA). Among groups, differences were evaluated using one-way ANOVA. Dunnett's *post-hoc* test was used to test for significant differences between each group compared with the untreated Het group, and a multiple comparison to find significant differences of EDL forces measured throughout the eccentric contraction was assessed using Bonferroni *post-hoc* test. Student's *t* test was used to determine cardiac MRI significant differences between the finerenone-treated Het and untreated Het group. Statistical significance was considered with *P* value ≤0.05.

Table 1 Preclinical study outcome measurements after 16 weeks of treatment with finerenone

	Control C57		Untreated Het		Finerenone treated		P value	
	mean ± SEM (n)	mean ± SEM (n)	mean ± SEM (n)	mean ± SEM (n)	ANOVA or Student's t test ^a	Dunnnett untreated vs. finerenone		
Body weight (g)	26.4 ± 0.7 (18)	28.1 ± 0.9 (18)	28.7 ± 0.8 (18)	28.7 ± 0.8 (18)	0.1152	0.8163		
Grip strength Trial 1 (N)	1.50 ± 0.05 (15)	1.00 ± 0.05 (15)	1.14 ± 0.04 (16)	1.14 ± 0.04 (16)	<0.0001	0.0265		
GS/BW Trial 1 (mN/g)	58.2 ± 1.5 (15)	35.2 ± 1.5 (15)	40.3 ± 1.0 (16)	40.3 ± 1.0 (16)	<0.0001	0.0182		
Grip strength Trial 5 (N)	1.5 ± 0.05 (15)	0.8 ± 0.04 (15)	1.1 ± 0.04 (16)	1.1 ± 0.04 (16)	<0.0001	0.0007		
GS/BW Trial 5 (mN/g)	56.5 ± 1.7 (15)	29.7 ± 1.1 (15)	37.5 ± 1.1 (16)	37.5 ± 1.1 (16)	<0.0001	0.0003		
Heart weight (g)	0.120 ± 0.004 (18)	0.12 ± 0.01 (18)	0.12 ± 0.01 (18)	0.12 ± 0.01 (18)	0.7587	1.0000		
HW/BW (mg/g)	4.5 ± 0.1 (18)	4.4 ± 0.2 (18)	4.3 ± 0.2 (18)	4.3 ± 0.2 (18)	0.6568	0.9321		
Heart rate (bpm)	696 ± 14 (17)	678 ± 15 (18)	704 ± 11 (18)	704 ± 11 (18)	0.3727	0.2893		
QT (ms)	46.0 ± 1.4 (17)	46.9 ± 1.1 (18)	45.8 ± 1.0 (18)	45.8 ± 1.0 (18)	0.7693	0.7277		
QTc (ms)	48.8 ± 1.0 (17)	49.3 ± 0.7 (18)	49.3 ± 0.7 (18)	49.3 ± 0.7 (18)	0.8570	0.9997		
Base peak systolic strain rate lateral (endocardial circumferential S ⁻¹)	ND	0.28 ± 0.03 (12)	0.39 ± 0.04 (11)	0.39 ± 0.04 (11)	0.0409 ^a			
Diaphragm specific force (mN/mm ²)	98.0 ± 11.0 (15)	151.8 ± 9.4 (18)	139.0 ± 8.2 (17)	139.0 ± 8.2 (17)	0.0007	0.5228		
EDL specific force (mN/mm ²)	431 ± 19 (16)	329 ± 13 (17)	318 ± 21 (17)	318 ± 21 (17)	<0.0001	0.8622		
Ecc1 (mN/mm ²)	400 ± 27 (17)	310 ± 18 (18)	298 ± 24 (18)	298 ± 24 (18)	0.0057	0.8965		
Ecc2 (% Ecc1)	98 ± 1 (17)	82 ± 2 (18)	94 ± 5 (18)	94 ± 5 (18)	0.0026	0.0186		
Ecc5 (% Ecc1)	86 ± 2 (17)	51 ± 4 (18)	71 ± 7 (18)	71 ± 7 (18)	<0.0001	0.0075		
Post-rest Ecc6 (% Ecc1)	90 ± 2 (17)	51 ± 4 (18)	72 ± 6 (18)	72 ± 6 (18)	<0.0001	0.0043		
Quad IgG (% CSA)	0.30 ± 0.04 (18)	5.6 ± 0.6 (18)	5.8 ± 0.6 (18)	5.8 ± 0.6 (18)	<0.0001	0.9644		
Heart IgG (% CSA)	0.3 ± 0.1 (18)	1.8 ± 0.3 (18)	1.1 ± 0.2 (18)	1.1 ± 0.2 (18)	0.0002	0.0796		

BW, body weight; CSA, percentage of cross-sectional area; GS, grip strength; HW/BW, heart weight normalized by body weight; QT, QT interval; QTc, QT adjusted for heart rate variability; EDL, extensor digitorum longus; Ecc, EDL force after eccentric contraction.

^aIndicates Student's t-test P values.

Measurements from treated (finerenone) and untreated dystrophic mice and wild type control mice (C57) are expressed as mean ± SEM. P values from ANOVA followed by Dunnett's post-hoc test are shown for 21 parameters. Underlined P values indicate statistical significance (P ≤ 0.05).

Results

To determine whether finerenone improved the earliest sign of cardiomyopathy detectable in mouse models and DMD patients, cardiac strain rate was measured using MRI. The

myocardial peak systolic strain rate at the base of the left ventricle showed a significant improvement ($P = 0.0409$) by a Student's t test comparing finerenone-treated Het mice ($0.39 \pm 0.04 \text{ s}^{-1}$) with untreated Het mice ($0.28 \pm 0.03 \text{ s}^{-1}$) (Table 1, Figure 1). Importantly, there was no statistically

Figure 1 Finerenone treatment improves cardiac strain rate at the base of left ventricle. Dot plot showing *in vivo* myocardial peak strain rate measured by magnetic resonance imaging in untreated Het ($n = 12$) vs. finerenone-treated Het mice ($n = 11$). Student's t test shows significant differences between groups ($*P = 0.0409$).

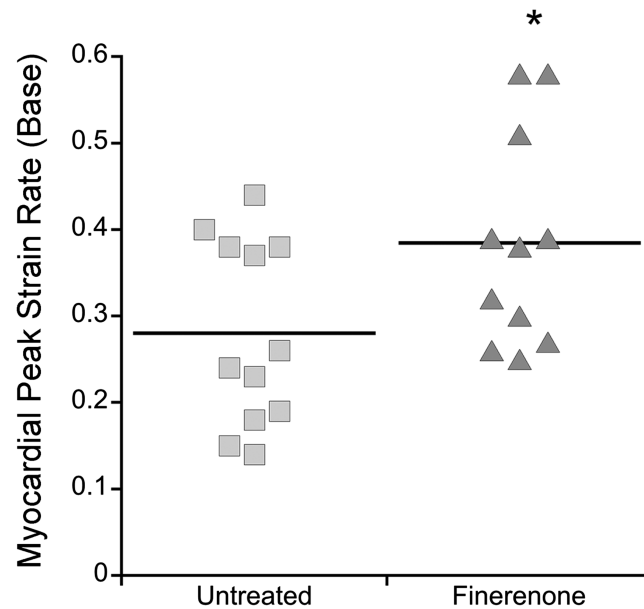
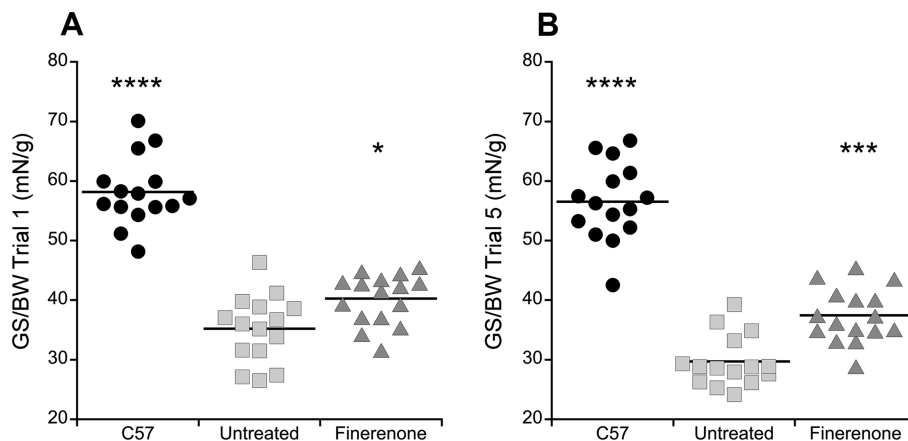


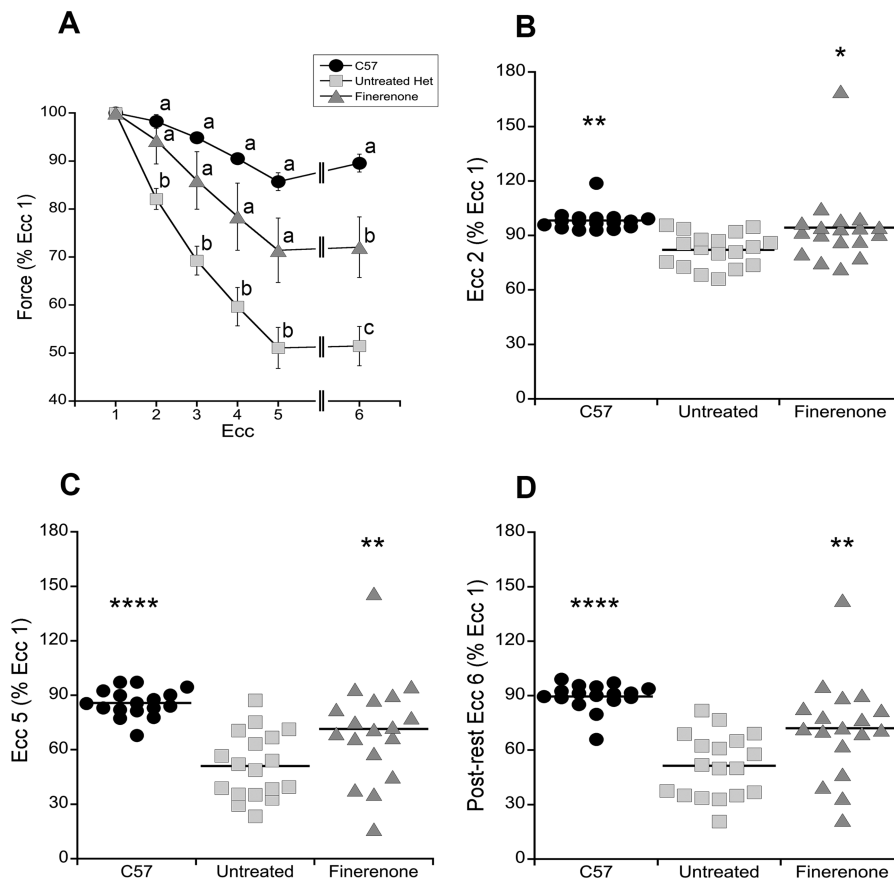
Figure 2 Finerenone improves normalized grip strength in Het mice at baseline and after fatigue. (A) Dot plot representing the highest peak force produced from rested mice during the first set of five pulls on a grip-strength meter. Significant differences in normalized grip strength were detected between groups by ANOVA ($P < 0.0001$), and then a Dunnett's *post-hoc* test was used to compare finerenone-treated Het mice and untreated Het mice and showed a significant improvement in treated mice ($P = 0.0182$). (B) Dot plot representing the highest peak force produced from fatigued mice during the fifth set of five pulls on a grip-strength meter. An even greater relative improvement was measured ($P = 0.0003$) in finerenone-treated mice compared to untreated Het mice. The force measured in wild-type (C57) mice is significantly larger compared with untreated het mice during Trial 1 ($****P < 0.0001$) and Trial 5 ($P < 0.0001$, Dunnett's *post-hoc* test) validating the assay. Treatment groups: C57 ($n = 15$), untreated ($n = 15$), Finerenone ($n = 16$). Statistical significance: $*P \leq 0.05$ and $***P \leq 0.001$ vs. untreated Hets. BW, body weight; GS, grip strength.



significant difference between the groups for the measured parameters of heart weight ($P = 0.7587$), heart weight normalized to body weight ($P = 0.6568$), or electrocardiographic parameters measured in conscious mice of heart rate ($P = 0.3727$), QT interval ($P = 0.7693$) or QTc adjusted for heart rate variability ($P = 0.8570$), which did not differ from previous published data³⁸ supporting the absence of negative consequences of drug treatment (Table 1). In addition, no evidence of arrhythmia was observed. Since Het mice model early stages of cardiomyopathy in humans with reduced strain rates, which occur years before reduced ejection fraction, both untreated and finerenone-treated groups retained normal whole heart function at 20 weeks-of-age with left ventricular ejection fractions of $73 \pm 2.7\%$ and $72 \pm 2.6\%$, respectively. Similarly, left ventricular end diastolic volumes were 0.05 ± 0.003 mL in finerenone-treated compared with

0.05 ± 0.004 mL in untreated Het mice ($P = 0.8320$) and end systolic volumes were 0.01 ± 0.002 mL in the finerenone-treated group compared with 0.03 ± 0.014 mL ($P = 0.3608$) in untreated Het mice. No differences between male and female mice were observed in any of the measured cardiac or skeletal muscle parameters. Because reduced overall muscle strength and fatigue are clinically relevant features of DMD that contribute to reduced quality of life, we next measured forelimb grip strength in a blinded manner to determine effects of finerenone treatment on the whole animal. The assay consisted of five repeats of five pulls on a grip-strength meter to determine the peak baseline grip strength during the first set and the peak grip strength after fatigue during the fifth set of pulls. Baseline total grip strength of finerenone-treated Het mice (1.14 ± 0.04 N) was improved ($P = 0.0265$) compared with untreated Het mice

Figure 3 Finerenone treatment improves *extensor digitorum longus* (EDL) force generation during a contraction induced injury protocol. (A) EDL forces measured throughout the eccentric contraction (Ecc) protocol as a percentage of force generation during the first eccentric contraction (Ecc1). Bonferroni *post-hoc* test was used to find significant differences throughout the eccentric contractions. Means that do not share a letter are significantly different from each other. (B–D) Measurements of EDL contractile force after Ecc2, Ecc5, and after a rest period (Ecc6) expressed as a percentage of force generated during Ecc1. The finerenone-treated group has significantly higher relative EDL contractile force compared with untreated Het mice after Ecc2 ($P = 0.0186$), Ecc5 ($P = 0.0075$), and after the rest period ($P = 0.0043$, Dunnett's *post-hoc* test). The same test was used to determine significant differences between wild-type (C57) and untreated Het mice after Ecc2 ($P = 0.0019$), Ecc5 ($P < 0.0001$), and after the rest period ($P < 0.0001$). Treatment groups: C57 ($n = 17$), untreated ($n = 18$), Finerenone ($n = 18$). Statistical significance: * $P \leq 0.05$, ** $P \leq 0.01$, and **** $P \leq 0.0001$.

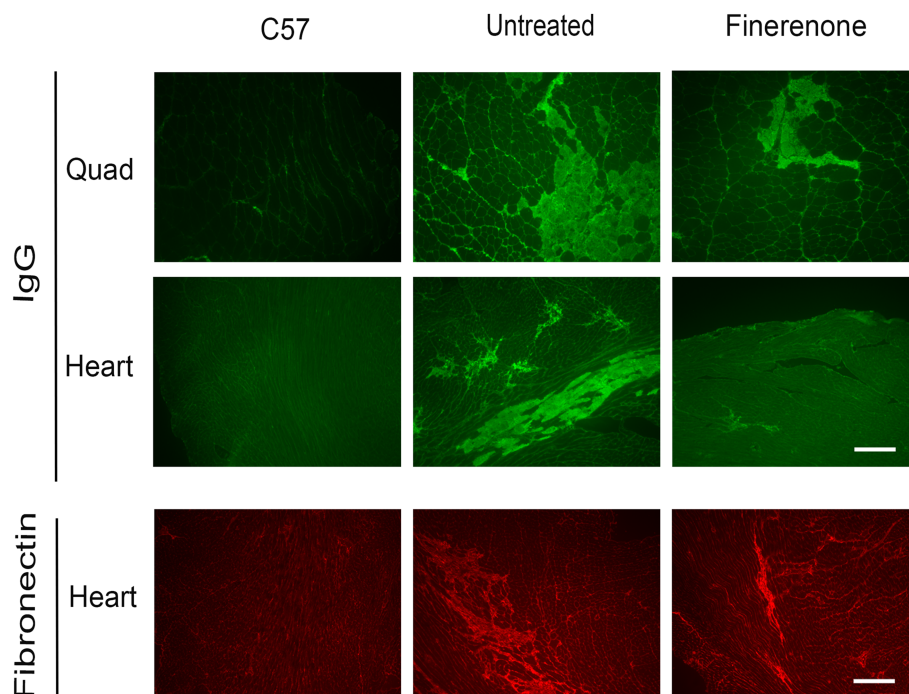


(1.00 ± 0.05 N) (Table 1). Grip strength normalized to body weight was also significantly increased in rested ($P = 0.0182$) finerenone-treated Het (40.3 ± 1.0 mN/g) compared with untreated Het (35.2 ± 1.5 mN/g) mice (Table 1; Figure 2). Furthermore, a greater relative improvement in normalized grip strength ($P = 0.0003$) was detected during the fifth trial in fatigued finerenone-treated Het mice (37.5 ± 1.1 mN/g) compared with untreated Het mice (29.7 ± 1.1 mN/g) (Table 1; Figure 2).

The absence of dystrophin in DMD patients and mouse models leads to an increased susceptibility of the muscle membrane to damage during activity, which can be experimentally measured as force reductions after lengthening or eccentric (Ecc) contractions of limb muscles.⁴⁰ Baseline EDL specific force was significantly different ($P < 0.0001$) between finerenone-treated Het mice (318 ± 21 mN/mm²), untreated Het mice (329 ± 13 mN/mm²), and wild-type control mice (431 ± 19 mN/mm²), but no significant differences were obtained when comparing finerenone-treated dystrophic Het mice with untreated Het mice ($P = 0.8622$, by Dunnett's *post-hoc* test) (Table 1). Similarly, there were no differences in specific force in diaphragm between finerenone-treated Het mice (139.0 ± 8.2 mN/mm²) compared with untreated Het mice (151.8 ± 9.4 mN/mm²) ($P = 0.5228$, by Dunnett's

post-hoc test) (Table 1). However, contraction-induced injury in EDL muscles from finerenone-treated Het mice was reduced resulting in higher specific force generation throughout the eccentric contraction protocol compared with untreated Het mice (Table 1; Figure 3A). This protocol serves as a strong differentiator between wild-type and dystrophic mice with damage accumulating in dystrophic muscles throughout the protocol (Figure 3A). During the second eccentric contraction, normalized tetanic force was significantly improved ($P = 0.0186$) in finerenone-treated Het mice ($94 \pm 5\%$ Ecc1) compared with untreated Het mice ($82 \pm 2\%$ Ecc1) (Table 1; Figure 3B). During the fifth eccentric contraction, normalized specific force was further separated between ($P = 0.0075$) finerenone-treated Het mice ($71 \pm 7\%$ Ecc1) compared with untreated Het mice ($51 \pm 4\%$ Ecc1) (Table 1; Figure 3C). To differentiate between contraction-induced accumulated damage and fatigue, normalized tetanic force was measured again after a rest period (Ecc6). Again, the finerenone-treated Het group ($72 \pm 6\%$ Ecc1) generated significantly higher ($P = 0.0043$) specific force compared with untreated Het mice ($51 \pm 4\%$ Ecc1), supporting that the prevention of force reduction was because of an effect of drug treatment on membrane stability and not fatigue (Table 1; Figure 3).

Figure 4 Representative images of immunofluorescence staining for serum IgG accumulation in quadriceps muscle and heart cross-sections and fibronectin in heart from wild-type (C57), untreated Het (untreated), and finerenone-treated Het (finerenone) mice. Serum IgG is excluded from healthy quadriceps and heart tissue but accumulates in muscle fibres with membrane damage (quadriceps) and in both damaged cardiomyocytes as well as fibrotic areas that have replaced cardiomyocytes in heart. Fibronectin staining confirms the presence of small fibrotic areas present in Het hearts. Wild-type control mice (C57) had no visible damage in either tissue. Data collected from 18 mice in each group: C57, untreated, and finerenone. Bar = 200 μ m.



To evaluate the effect of finerenone on ongoing baseline muscle damage in dystrophic skeletal muscles and on accumulated and ongoing damage in heart, we also quantified the presence of serum IgG immunofluorescence within these tissues (Table 1). Quantification of IgG positive cross-sectional area in quadriceps and heart muscle from wild-type control (C57), untreated Het and finerenone-treated Het mice revealed significant differences in the amount of damage ($P < 0.0001$ and $P = 0.0002$, respectively). Dunnett's *post-hoc* test showed no differences between finerenone-treated ($5.8 \pm 0.6\%$) and untreated Het ($5.6 \pm 0.6\%$) mice in the amount of IgG immunofluorescence in quadriceps, which were both quite low. However, hearts from finerenone-treated Het mice ($1.1 \pm 0.2\%$) showed a trend towards less accumulated damage than untreated Het mice ($1.8 \pm 0.3\%$). Fibronectin immunofluorescence was used to show that most of this low percentage of IgG staining in heart was because of its accumulation in fibrotic tissue that had already replaced cardiac muscle (Figure 4).

Previous studies have investigated the effect of MR-regulated gene expression in the heart in models of advanced induced heart failure.^{32,41,42} However, in this study,

finerenone is able to prevent very early functional changes in cardiomyopathy progression detectable as cardiac strain rate abnormalities, which occur in DMD mouse models and patients far before reduced whole heart function. Therefore, we decided to assess gene expression changes in finerenone-treated Het mice compared with untreated Het mice to identify potential very early cardiomyopathic changes that are targeted by non-steroidal MR antagonism. We conducted a gene expression microarray analysis and observed that expression of 19 genes was increased and expression of eight genes was decreased (Table 2). Gene networks represented in the differentially expressed genes included circadian rhythm and ubiquitination (Table 2).

Discussion

This study is the first to demonstrate that treatment with the non-steroidal MRA drug finerenone alone is able to improve the clinically relevant earliest diagnostic parameter of cardiomyopathy in DMD patients, cardiac strain rate.^{21,43,44} In addition, finerenone is able to improve skeletal muscle strength

Table 2 Gene expression microarray differences greater than two-fold in ventricular heart tissue from finerenone-treated Het vs. untreated Het mice

Fold change (treated/untreated)	Gene symbol	Full gene name	Function
3.01	<i>Wee1</i>	WEE 1 homologue 1	Protein banding
2.89	<i>Gm4899</i>	Predicted gene 4899	Cellular respiration, ubiquinone binding
2.87	<i>Per2</i>	Period circadian clock 2	Circadian regulation of gene expression
2.44	<i>Gm561</i>	Predicted gene 561	Unknown function
2.27	<i>Lrrc52</i>	Leucine rich repeat containing 52	Ion transport
2.17	<i>Alox5ap</i>	Arachidonate 5-lipoxygenase activating protein	Protein banding
2.16	<i>Gm10260</i>	Predicted gene 10260	Translation
2.16	<i>Rps27a-ps2</i>	Ribosomal protein S27A, pseudogene 2	Ubiquinone binding
2.14	<i>Gm11689</i>	Predicted gene 11689	Unknown function
2.13	<i>Rps27l</i>	Ribosomal protein S27-like	Ion binding, apoptosis, translation
2.11	<i>Coq10b</i>	Coenzyme Q10 homologue B	Cellular respiration, ubiquinone binding
2.08	<i>Mrpl41</i>	Mitochondrial ribosomal protein L41	Translation
2.08	<i>Gm8430</i>	Predicted pseudogene 8430	Ubiquinone binding
2.06	<i>Bst2</i>	Bone marrow stromal cell antigen 2	Immune system process, transmembrane
2.05	<i>Wbp5</i>	WW domain binding protein 5	Regulation of transcription
2.03	<i>Ier3</i>	Immediate early response 3	Protein banding, regulation of DNA repair, transmembrane, apoptosis
2.02	<i>Per3</i>	Period circadian clock 3	Circadian regulation of gene expression
2.01	<i>Gm2000</i>	Predicted gene 2000	Ribosomal protein
2	<i>Il33</i>	Interleukin 33	Transcription
-2.03	<i>Clock</i>	Circadian locomotor output cycles kaput	Circadian regulation of gene expression
-2.04	<i>Adam19</i>	A disintegrin and metallopeptidase domain 19 (meltrin beta)	Protein banding
-2.2	<i>Mir467e</i>	MicroRNA 467e	Micro-ribonucleoprotein complex
-2.3	<i>Lbh</i>	Limb-bud and heart	Regulation of transcription, regulation of blood coagulation
-2.59	<i>Zfp36</i>	Zinc finger protein 36	Regulation of cytokine production/immune response
-3.96	<i>Slc41a3</i>	Solute carrier family 41, member 3	Protein banding
-4.85	<i>Cdkn1a</i>	Cyclin-dependent kinase inhibitor 1A (P21)	Apoptosis, protein banding
-5.71	<i>Arntl</i>	Aryl hydrocarbon receptor nuclear translocator-like	Circadian regulation of gene expression

and muscle membrane stability in this muscular dystrophy model. Finerenone in comparison with commonly used previous steroidal antagonists, spironolactone and eplerenone, combines high potency and selectivity. Previous preclinical and clinical studies have shown equivalent efficacy of spironolactone and eplerenone added to an ACEi or ARB in DMD mouse models and patients, but efficacy for MRA used alone has not been demonstrated for heart or skeletal muscles.^{14,15,21,23} MRAs tend to cause less hypotension than ACEi or ARB, so MRAs alone, without these additional drugs, may be advantageous as a therapy for DMD patients who are typically normotensive. In cardiac clinical trials in DMD patients, spironolactone nor eplerenone added to ACEi or ARB reduced blood pressure nor changed salt balance from baseline over 3 years compared to standard therapy alone.^{21–23} Typical measurements of renal function by urine or serum creatine are not used in DMD because of the large amount of muscle breakdown, but cystatin C has been used to demonstrate normal renal function in DMD patients.^{45,46} Therefore, although finerenone results in less worsening of renal function in chronic kidney disease,³¹ specific investigations of renal function were not included in the present study.

Finerenone led to overall increased baseline total and normalized grip strength, a measure of whole body muscle force that is reduced by dystrophin-deficiency and a measure of muscle function that is relevant for patients with DMD. A major symptom experienced by DMD patients is fatigue due to their muscle weakness. Finerenone led to even greater significant improvements in normalized grip strength after five repeats of the grip strength protocol, which resulted in lower forces in untreated dystrophic mice compared with baseline measurements. This improvement in grip strength correlates with improved muscle forces observed in dystrophic mice with a conditional knockout allele of the MR specifically in skeletal muscle fibres.²⁴

Muscles lacking dystrophin are highly susceptible to damage during use. The typical state-of-the-art preclinical measurement of this contraction-induced injury is to record force throughout a repetitive lengthening contraction protocol in EDL.⁴⁷ In addition to improvements in muscle force and fatigue, finerenone also contributed to prevention of force drop during this protocol, supporting the ability of this treatment to partially prevent muscle injury. This observation was consistent with the direct membrane stabilizing effect of spironolactone we have previously demonstrated in a different assay that uses a laser to measure membrane stability, which is also greatly compromised in dystrophic muscles.²⁴ The ability of finerenone to prevent muscle damage is a key therapeutic effect for treating both the cardiac and skeletal muscle dysfunction in muscular dystrophies. Although this study demonstrates that finerenone prevents reduced cardiac strain rate, the earliest detectable functional sign of DMD cardiomyopathy in both mouse models and patients,

it does not address the longevity of these effects, because of the lack of any DMD model that reproducibly progresses into heart failure. Mice lacking both copies of utrophin in addition to dystrophin (*utrn*^{-/-};*mdx*) have a more severe cardiomyopathy in terms of fibrosis and reduced function than the *utrn*^{+/-};*mdx* Het mice used here. However, half of utrophin/dystrophin-deficient mice die from their skeletal muscle pathology by 10 weeks-of-age and the remainder by 20 weeks-of-age,⁴⁸ prohibiting comparison with untreated controls in chronic treatment studies such as that described here. Future studies using cell-type specific knock-out mouse models and improved models of DMD heart failure will be needed to separate the contribution from possible mechanisms to the cardiac benefits of MRAs in muscular dystrophy.

The presence of high levels of aldosterone synthase in myeloid cells from dystrophic skeletal muscles will need to be confirmed in cardiac pathology to determine whether blocking signalling from chronic inflammation contributes to finerenone's mechanism of action in heart.²⁰ Finerenone has previously been shown to reduce myocardial reactive oxygen species (ROS) in Zucker rats, a rat model of metabolic syndrome.⁴⁹ Because ROS also contributes to dystrophic cardiac pathology, this pathway may also contribute to finerenone's efficacy in this model.^{50,51} The mechanisms underlying finerenone's improvement of skeletal muscle and cardiac muscle parameters are likely multi-factorial, involving a combination of effects on membrane stabilization, mechanical force, and MR-regulated gene expression changes in multiple cell types present in the dystrophic muscle microenvironment that have direct or indirect anti-fibrotic effects.

Finerenone did not lead to a large number of persisting gene expression changes in ventricular heart tissue at the end of the treatment compared with untreated dystrophic hearts. A surprising finding was the presence of four genes involved in circadian rhythm. Finerenone treatment in dystrophic hearts increases *Per2* and *Per3*, which compose the negative arm of the circadian clock, and decreases *Arntl* and *Clock*, which compose the positive arm of the clock.⁵² These data suggest that finerenone has shifted or enhanced the amplitude of the clock. Disruption of the circadian clock in heart has been previously demonstrated to change the expression of numerous downstream inflammatory and fibrotic genes.⁵³ In our previous studies, of these genes, only *Per3* was increased in quadriceps muscles of mice treated with lisinopril and spironolactone, but other genes associated with circadian rhythm, *Dbp* and *Nr1d1*, were also increased.¹³ *Per3* was increased by the addition of spironolactone to aldosterone treatment of normal human myotubes in culture.³⁹ Treatment of H9c2 cardiomyoblast-like cells with aldosterone has previously been shown to regulate *Per1* and initiate the cycling of *Per2* and *Arntl* in these non-endogenously cycling cells.^{53,54} Myeloid MR conditional knockout mice

demonstrated a reduction in the circadian rhythm of blood pressure.^{53,55} These data suggest that MR antagonism may cause alterations in circadian rhythm that contribute to its beneficial effect on function, but further carefully timed studies will need to be carried out to determine the amplitude and frequency of expression of these oscillating genes.⁵²

A limitation of this gene expression data is that because of the extensive preclinical testing of each individual mouse, mice were sacrificed and heart tissue collected at two different times of the day: 10 a.m. and 1 p.m., because only two mice per day could be measured for muscle physiology. It is therefore possible that circadian rhythm gene expression differences with finerenone treatment are a side effect of dissection of tissues at different times of the day. All three of the untreated Het mice used for gene expression microarray were dissected at approximately 10 a.m., while one of the finerenone-treated group was dissected at 10 a.m. and the other two were dissected at 1 p.m. Future mechanistic studies will need to address whether alterations in the heart clock are sufficient for improving dystrophic cardiomyopathy compared with other possible mechanisms of finerenone's function.

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Acknowledgements

We would like to thank Anna Bratasz and Michelle Williams for conducting the MRI scans in the Small Animal Imaging Core at the Davis Heart and Lung Research Institute. The authors also thank Paolo Fadda and Huabao Wang for microarray processing and their technical guidance at the Ohio State Genomic Shared Resource-Comprehensive Cancer Center, microarray core. We also would like to thank Stefanie Breitenstein, Bayer AG clinical development, for discussion and support.

Conflict of Interest

This study was funded by Bayer AG, Wuppertal, Germany; PS and PK are full-time employees of Bayer AG, all other authors have declared that no conflict of interest exists.

Funding

This work was funded by Bayer AG, Wuppertal, Germany.

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