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**CONTRACTILE EFFICIENCY OF DYSTROPHIC MDX MOUSE MUSCLE: IN VIVO AND EX VIVO ASSESSMENT OF ADAPTATION TO EXERCISE OF FUNCTIONAL END-POINTS**

Roberta Francesca Capogrosso<sup>1, 5</sup>, Paola Mantuano<sup>1</sup>, Anna Cozzoli<sup>1</sup>, Francesca Sanarica<sup>1</sup>, Ada Maria Massari<sup>1</sup>, Elena Conte<sup>1</sup>, Adriano Fonzino<sup>1</sup>, Arcangela Giustino<sup>2</sup>, Jean-Francois Rolland<sup>1</sup>, Angelo Quaranta<sup>3</sup>, Michela De Bellis<sup>1</sup>, Giulia Maria Camerino<sup>1</sup>, Robert W. Grange<sup>4</sup>, Annamaria De Luca<sup>1</sup>.

<sup>1</sup>Section of Pharmacology, Department of Pharmacy & Drug Sciences, University of Bari "Aldo Moro", 70125 Bari, Italy.

<sup>2</sup>Department of Biomedical Sciences and Human Oncology, School of Medicine, University of Bari "Aldo Moro" Bari, Italy

<sup>3</sup>Department of Veterinary Medicine, University of Bari "Aldo Moro", 70010 Valenzano (BA), Italy

<sup>4</sup>Department of Human Nutrition, Foods, and Exercise, Virginia Tech University, Blacksburg, VA 24061, USA

<sup>5</sup>Department of Chemical, Toxicological and Pharmacological Drug Studies, Catholic University  
“Our Lady of Good Counsel”, Tirana, Albany

**Running head:** Effect of exercise on muscle contraction of dystrophic mice

**Corresponding author**

Prof. Annamaria De Luca

Sezione di Farmacologia

Dipartimento di Farmacia – Scienze del Farmaco

Università degli Studi di Bari “Aldo Moro”

email: [annamaria.deluca@uniba.it](mailto:annamaria.deluca@uniba.it)

tel (+39) 080 5442245

fax(+39) 080 5442801

## **ABSTRACT**

Progressive weakness is a typical feature of Duchenne muscular dystrophy (DMD) patients and is exacerbated in the benign mdx mouse model by in vivo treadmill exercise. We hypothesized a different threshold for functional adaptation of mdx muscles in response to the duration of the exercise protocol. In vivo weakness was confirmed by grip strength after 4, 8 and 12 weeks of exercise in mdx mice. Torque measurements revealed that exercise-related weakness in mdx mice correlated with the duration of the protocol, while wild-type (wt) mice were stronger. Twitch and tetanic forces of isolated diaphragm and extensor digitorum longus (EDL) muscles, were lower in mdx compared to wt mice. In mdx, both muscle types exhibited greater weakness after a single exercise bout, but only in EDL after a long exercise protocol. As opposite to wt muscles, mdx EDL ones did not show any exercise-induced adaptations against eccentric contraction force drop.

qRT-PCR analysis confirmed the maladaptation of genes involved in metabolic and structural remodeling, while damage-related genes remained significantly upregulated and angiogenesis impaired. Phosphorylated AMP kinase level increased only in exercised wt muscle. The severe histopathology and the high levels of muscular TGF- $\beta$ 1 and of plasma matrix metalloproteinase-9 confirmed the persistence of muscle damage in mdx mice. Then, dystrophic muscles showed a partial degree of functional adaptation to chronic exercise, although not sufficient to overcome weakness nor signs of damage. The improved understanding of the complex mechanisms underlying maladaptation of dystrophic muscle paves the way to a better management of DMD patients.

## **NEW & NOTEWORTHY**

We focused on the adaptation/maladaptation of dystrophic mdx mouse muscles to a standard protocol of exercise to provide guidance in the development of more effective drug and physical therapies in DMD. The mdx muscles showed a modest functional adaptation to chronic exercise, but it was not sufficient to overcome the progressive in vivo weakness, nor to counter signs of

muscle damage. Then a complex involvement of multiple systems underlies the maladaptive response of dystrophic muscle.

## **KEY WORDS**

Duchenne muscular dystrophy, mdx mouse model, treadmill exercise, in vivo and ex vivo weakness, isometric and eccentric contraction.

## INTRODUCTION

Properly prescribed exercise is pivotal to maintain general health and has multiple therapeutic benefits in metabolic and cardiovascular disorders. Exercise is also the main regulator of skeletal muscle plasticity. Mechano-sensitive signaling is modulated by intensity, frequency and duration of muscle activity that yields adaptations in function, morphology and metabolism in relation to demand. However, exhausting or unaccustomed exercise may lead to metabolic disturbance, inflammation and damage in skeletal muscle of healthy subjects (22, 30). The delicate equilibrium between positive and harmful muscle effects of exercise is compromised in progressively degenerating myopathies, such as Duchenne muscular dystrophy (DMD), with important implications for setting appropriate physical therapies (7, 37). DMD is a severe muscle-wasting disease affecting about 1 in 5000 boys and is caused by mutations of the dystrophin gene on the X chromosome (25). Dystrophin is a subsarcolemmal protein that is a key component of the dystrophin-glycoprotein complex (DGC) and acts as a ‘shock absorber’ at the cytoskeleton-membrane-extracellular matrix interface, via domain-specific interaction with actin, microtubules and  $\beta$ -dystroglycan. In addition, through the interaction with  $\alpha$ -syntrophin and neuronal nitric oxide synthetase (NOS), it modulates proper NO production and vasodilation of muscle during contraction (1, 25, 29, 33, 39).

The absence of dystrophin leads to disorganization of the DGC and triggers a complex pathogenic cascade leading to progressive myofiber death and fibrosis. Serious functional consequences are weakness, loss of ambulation and respiratory dysfunction rapidly progressing and finally leading to death around thirty (7, 25). The use of animal models, such as the widely used mdx mouse, highlighted mechanisms underlying the progressive myofiber necrosis, including mechanical fragility of the sarcolemma, aberrant calcium homeostasis, mitochondrial distress, unbalanced oxidative stress and chronic inflammation (1, 21, 42).#

Many alterations due to the absence of dystrophin are related to the role of the protein in mechano-transduction (6), focusing on possible altered responses of dystrophic muscle to exercise. Exercise regimens exert beneficial and harmful effects in mdx mice in a narrow range depending on the duration, pattern and intensity of the protocol used (5, 16, 23, 46, 54). Voluntary exercise, such as swimming or wheel running, seems to delay the progression of pathology; however, muscle specific responses to a mechanical challenge may occur in relation to mouse age and duration of the training period (8, 16, 23, 26). For instance, Selsby et al., found that long-term volitional wheel running improves function of hind limb and cardiac muscles, while impairing those of diaphragm, in line with the proposed susceptibility of the latter to continuous mechanical respiratory challenge (42, 48, 49). In contrast, forced chronic exercise or downhill running imposes harmful effects in the mdx phenotype (5, 12, 14, 38, 46, 54); accordingly, forced protocols of chronic treadmill exercise are considered a feasible strategy to better assess the efficacy of pharmacological interventions in the mdx mouse model (12, 13, 15, 21, 56). We have widely demonstrated that at least 4 weeks of a standard protocol of exercise on a horizontal treadmill worsens in vivo weakness and fatigue in mdx but not in wild-type (wt) mice, allowing a non-invasive longitudinal evaluation of drug outcome (5, 11, 12). Signs of increased muscle damage have been observed at the histological and biochemical level, in parallel with an increase in calcium entry in myofibers via mechanosensitive channels in the exercised mdx muscles (5, 12, 17, 47). We have recently shown that our protocol of chronic treadmill exercise also exacerbates a failing mechanical-metabolic coupling of dystrophic muscle. In particular, exercise-sensitive genes involved in protective metabolic pathways or autophagy such as sirtuin-1 and peroxisome proliferator-activated receptor  $\gamma$  coactivator 1- $\alpha$  (Sirt1/Pgc-1 $\alpha$ ), peroxisome proliferator-activated receptor  $\gamma$  (Ppar $\gamma$ ), adiponectin, Bcl-2 nineteen-kilodalton interacting protein 3 (Bnip-3) are all severely down-regulated in a time-dependent manner in gastrocnemius of exercised dystrophic mice (9). In addition, damage-related genes such as NADPH-oxidase 2 (Nox2), transforming growth factor-beta1 (Tgf- $\beta$ 1), tumor necrosis factor alpha (Tnf- $\alpha$ ), and c-Src tyrosine kinase, remain severely upregulated, with a consequent reinforcement of

signals for muscle damage and dysfunction (9). However, the impact of our chronic exercise protocol on mdx muscle contractile function, i.e. assessed via nerve stimulation in anesthetized animals or as efficiency of contractile machinery in isolated muscles, has not yet been explored. We hypothesize that dystrophic myofibers have a different mechanical and/or time-dependent threshold for functional adaptation to exercise compared to wt muscle. The verification of the above hypothesis and of the underlying mechanisms may have important consequences for therapeutic and physical management of DMD patients. To this end, herein we assessed the change in muscular strength in response to chronic treadmill exercise protocols of either short (4-6 weeks) or long (> 12 weeks) duration in both mdx and wt mice. Outcome measures evaluated in vivo included grip strength and torque, and ex vivo the contractile properties of diaphragm and extensor digitorum longus (EDL) muscles. In parallel, biomarkers of muscle damage were also monitored by means of histology and biochemical approaches. The possible occurrence of long-term adaptation of contractile function to physical activity was evaluated by assessing the outcome of an acute single exercise session on ex vivo muscle force.

Our overall purpose was to gain insight into the equilibrium between adaptation and maladaptation of contractile efficiency of dystrophic muscle in response to exercise and thereby guide better management of the progressive muscle weakness of DMD patients.

## **MATERIALS AND METHODS**

All experiments were conducted in conformity with the Italian law for Guidelines for Care and Use of Laboratory Animals (D.L. 116/92), and European Directive (2010/63/UE).

Most of the experimental procedures used, conform to the standard operating procedures for pre-clinical tests in mdx mice available on the TREAT-NMD website (<http://www.treat-nmd.eu/research/preclinical/dmd-sops/>).

### **In vivo studies**

#### *Experimental groups and protocols*

Male wild-type (wt, C57/BL10) and mdx mice (C57/BL10ScSn*Dmd*<sup>mdx</sup>) at an initial age of 4-5 weeks were used for this study (Charles River, Italy for Jackson Laboratories, USA). For in vivo and ex vivo experiments, a total of 53 wt and 55 mdx mice were used. The wt and mdx mice were divided into different experimental groups. Within each experimental group mice were further divided into sedentary and exercised animals.

The exercise consisted of 30 min running on an horizontal treadmill (Columbus Instruments, USA) at 12meter/min, twice a week with a consistent 48h or 72h break between each exercise session (11, 12, 14). Sedentary mice could freely move in the cage (cage-based activity), but were provided no additional exercise. Three groups of mice were defined on the basis of age and duration of exercise/protocol: Group 1 was composed of 20 mdx mice (9 exercised and 11 sedentary) and 22 wt mice (8 exercised and 14 sedentary). The exercised mice underwent a “short protocol” of exercise (~4 weeks); all animals of group 1 were exclusively used for ex vivo physiology measurements. Group 2 was composed of 20 mdx (10 exercised and 10 sedentary) and 18 wt (10 sedentary and 8 exercised). In this group the exercised mice underwent a “long protocol” of exercise (> 12 weeks). All mice of group 2 were monitored weekly for body mass and fore-limb strength. The latter was measured with a grip strength meter (Columbus Instruments, USA), according to standard procedures (10). These measurements were performed at the beginning (T0), and after 4 (T4), 8 (T8) and 12 (T12) weeks of either exercise or cage-based activity. At the same time points, the in vivo hindlimb torque of all animals in Group 2 were also assessed (see below). Based on the in vivo results, which demonstrated remarkable time-dependent effects of exercise, Group 2 mice were exercised for up to 20 weeks (an additional 8 weeks) before the ex vivo experiments (long protocol). Cage-based activity mice were assessed at the same time points.

At the end of the short (4 weeks) or long (20 weeks) exercise protocols, ex vivo experiments were conducted. The mice continued to exercise until the day of sacrifice. Due to the time required to complete each ex vivo experiment, only two mice/day were assessed. Therefore, for mice of group 1, the mean duration of exercise was  $5.5 \pm 0.42$  weeks for wt and  $6.78 \pm 0.22$  weeks for mdx, with a



mean mouse age of  $11.5 \pm 0.42$  and  $11 \pm 0.3$  weeks, respectively; for group 2, the duration of exercise was 21 weeks for wt and 22 for mdx mice, with a mean mouse age of  $25.13 \pm 2.02$  and  $26.80 \pm 1.8$  weeks, respectively. For simplicity, throughout the text, the two time points are identified as the short and long exercise protocols. The protocol time lines and endpoints for mice of groups 1 and 2 are shown in Fig.1.

A last group of mice, identified as group 3, was composed of mdx and wt mice at 16 weeks of age. These mice were used to evaluate the acute effects of exercise. Five animals per genotype underwent a single bout of 30 min treadmill running as described above and were sacrificed 24 hours later to assess ex vivo contractile properties. These data were compared to age-matched non-exercised animals.

### *In Vivo Torque*

In vivo maximal isometric torque of the plantarflexor muscle group (gastrocnemius and soleus muscles) was assessed at the various time points of chronic exercise or cage-based activity. Mice were anaesthetized via inhalation ( $\sim 4\%$  isoflurane and  $1.5\%$   $O_2$  l/min) and placed on a thermostatically controlled table; anesthesia was maintained via nose-cone ( $\sim 2\%$  isoflurane and  $1.5\%$   $O_2$  l/min). The right hindlimb was shaved and aseptically prepared, and the foot was placed on the pedal connected to a servomotor (Model 300C-LR; Aurora Scientific, Aurora, Ontario, Canada). Contractions were elicited by percutaneous electrical stimulation of the tibial nerve via needle electrodes (Chalgren Enterprises Inc.) connected to a stimulator (Model 701B; Aurora Scientific, Aurora, Ontario, Canada) to induce contraction of the group of plantarflexor muscles. The current was adjusted from 30 to 50 mA until maximal isometric torque was achieved. A series of stimulations were then performed at increasing frequencies: 1, 10, 30, 50, 80, 100, 120, 150, 180, 200 Hz with a pulse train of 200 ms. Data were analyzed using Dynamic Muscle Analysis software (DMAv5.201; Aurora Scientific) to obtain torque, that was normalized to mouse body weight. Normalized values were used to construct torque-frequency curves.

## **Ex vivo studies**

### **Muscle preparation**

Animals were anesthetized with ketamine (100 mg/kg, i.p.) combined with xylazine (16 mg/kg, i.p.). If necessary, half the dosage of only ketamine was administered to mice to obtain a deep plane of anesthesia. Strips of right hemi-diaphragm and the extensor digitorum longus (EDL) muscle of one hind limb were removed and rapidly placed in the recording chambers for isometric and eccentric contraction measurements. Gastrocnemius (GC) muscles from one hind limb were dissected from surrounding tissue and were snap frozen in isopentane cooled in liquid nitrogen until processed for histological analysis. The contralateral EDL, tibialis anterior (TA) and gastrocnemius muscles were removed, washed in phosphate buffered saline (PBS), rapidly frozen in liquid nitrogen and stored at -80°C until used for qRT-PCR, western blot and ELISA assays, respectively.

### **Isometric and eccentric contractions**

For contractile recordings, EDL muscles and diaphragm strips were securely tied at the tendon insertion and placed in a muscle chamber containing a normal physiological salt solution (in mM): NaCl 148; KCl 4.5; CaCl<sub>2</sub> 2.0; MgCl<sub>2</sub> 1.0; NaHCO<sub>3</sub> 12.0; NaH<sub>2</sub>PO<sub>4</sub> 0.44 and glucose 5.55, continuously gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub> (pH = 7.2-7.4; 27±1 °C). One tendon was fixed to a force transducer (FORT25, WPI, Inc., FL, USA or a 1N 300C-LR, Aurora Scientific Inc. Canada) connected to proper interface and acquisition units (TCI 102 interface and an MP 100 acquisition unit; Biopac Systems, Santa Barbara, CA, USA or Dynamic Muscle Control Acquisition System v.5.415 ASI), while the opposite tendon was fixed to a chamber hook. Electrical field stimulation was obtained by two platinum electrodes connected to a stimulator (LE 12406, 2Biological Instruments, VA, Italy) that closely flanked the muscle or the diaphragm strips. After equilibration (30 minutes), the preparation was stretched to its optimal length (L<sub>0</sub>; measured with calipers), i.e. the length producing the maximal twitch in response to a 0.2 ms square wave 40-60 V pulse. A

force frequency relationship was obtained with trains of 350 ms (for EDL) and 450 ms (for diaphragm) duration over a range of frequencies (10-140 Hz). Fatigue was recorded with 2 min trains of 100 Hz tetani under isometric conditions and measured as the percentage of force reduction at the 5<sup>th</sup> pulse compared to the first pulse. Full recovery from fatigue was assessed before starting the eccentric contraction protocols; recovery occurred in about 20 minutes for both wt and mdx muscles.

For measuring eccentric contraction force drop, a train of 10 consecutive eccentric contractions were elicited, each separated by 30s. Muscles were stimulated at 120 Hz for 500 ms. Specifically, an isometric contraction was elicited by a 300 ms stimulation, followed by a stretch of 10%  $L_0$  at a speed of  $1L_0\text{ s}^{-1}$  for the following 200 ms. The progressive decay in isometric force vs. the initial contraction was measured as percent reduction. Single tetanic isometric contractions recorded at 4 and 30 min after the train were elicited to estimate muscle recovery from the stretch-induced force drop compared to the tetanic force before the eccentric contraction protocol (57).

Data were analyzed using AcqKnowledge software v. 3.8 (Biopac System) and/or Dynamic Muscle Analysis Software v.5.201 (ASI), to obtain maximal twitch and tetanic tensions, contraction kinetics and to construct force-frequency curves. At the end of the experiments, tendons were removed and the muscles were blotted dry and mass determined. Absolute values of tension were normalized by cross sectional area according to the equation  $sP = P/(\text{Mass}/L_f \cdot D)$  where P is absolute tension; Mass is the muscle mass; D is the density of skeletal muscle ( $1.06\text{ g/cm}^3$ );  $L_f$  was determined by multiplying  $L_0$  by previously reported muscle length to fiber length ratios: 0.44 for the EDL and 1 for the diaphragm (3, 5).

### **Isolation of total RNA, reverse transcription and Real-time PCR**

For each EDL muscle, total RNA was isolated by RNeasy Fibrous Tissue Mini Kit (Quiagen C.N. 74704) and quantified by spectrophotometry (ND-1000 NanoDrop, Thermo Scientific). To perform reverse transcription for each sample, 400 ng of total RNA was added to 1  $\mu\text{l}$  dNTP mix at 10 mM,

(Roche N.C. 11277049001), 1  $\mu$ l Random Hexamers 50  $\mu$ M (life-technologies C.N. n808-0127) and incubated at 65°C for 5 min. Afterward, 4  $\mu$ l 5X First Standard Buffer (life-technologies C.N. Y02321), 2  $\mu$ l 0.1 M DTT (life-technologies C.N. Y00147) and 1 $\mu$ l Recombinant RNasin Ribonuclease Inhibitor 40 U/ $\mu$ l (Promega, C.N. N2511) were added and incubated at 42°C for 2 min. Then, 1  $\mu$ l Super Script II Reverse Trascriptase 200 U/ $\mu$ l (life-technologies C.N. 18064-014) was added and samples were incubated at 25°C for 10 min, at 42°C for 50 min and at 70°C for 15 min. Real-time PCR was performed in triplicate using the Applied Biosystems Real-time PCR 7500 Fast system, MicroAmp Fast Optical 96-Well Reaction Plate 0.1 mL (Life-Technologies C.N. 4346906) and MicroAmp Optical Adhesive Film (life-technologies C.N. 4311971). Each reaction was carried in triplicate on a single plex reaction. Reactions consisted of 8 ng of cDNA, 0.5  $\mu$ l of TaqMan Gene Expression Assays, (life-technologies), 5  $\mu$ l of TaqMan Universal PCR master mix No AmpErase UNG (2x) (Life-Technologies C.N. 4324018) and Nuclease-Free Water not DEPC-Treated (Life-Technologies C.N. AM9930) for a final volume of 10  $\mu$ l. RT-TaqMan-PCR conditions were: step 1: 95°C for 20 s; step 2: 95°C for 3 s; and step 3: 60°C for 30 s; steps 2 and 3 were repeated 40 times. The results were compared with a relative standard curve obtained from 5 points of 1:4 serial dilutions. The mRNA expression of the genes was normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Gapdh). TaqMan Hydrolysis primer and probe gene expression assays were ordered with the following Assay IDs: glyceraldehyde-3-phosphate dehydrogenase (Gapdh), Assay IDs: Mm99999915\_g1; Peroxisome proliferative activated receptor gamma coactivator 1  $\alpha$  (Pgc-1 $\alpha$ ), Assay IDs: Mm01208835\_m1; Sirtuin 1 (Sirt1), Assay IDs: Mm00490758\_m1; myosin heavy chain 1 (Myh1), Assay IDs: Mm00600555\_m1; follistatin (Fst), Assay IDs: Mm00514982\_m1; NADPH oxidase 2 (Nox2), Assay IDs: Mm01287743\_m1; tubulin  $\alpha$ -1b (Tuba1b), Assay IDs: Mm01964369\_g1; cluster of differentiation 31 (Cd31), Assay IDs: Mm 01242576\_m1; factor inhibiting hypoxia-inducible factor (HIF)-1 (Fih1), Assay IDs: Mm 01198376\_m1; Integrin subunit alpha 7 (Itga7), Assay IDs: Mm 00434400\_m1; collagen, type I, alpha 1 (Colla1), Assay IDs: Mm 00801666\_g1; collagen type III

alpha 1 (Col3a1), Assay IDs: Mm 01254476\_m1; Elastin (Eln), Assay IDs: Mm 00514670\_m1; Acetylcholine receptor subunit alpha 1 (Achr-1), Assay IDs: Mm 00431629\_m1.

### **Protein expression analysis by western blot**

Protein extractions and immunoblots for the determination of phosphorylated AMPK/AMPK were performed (50). Briefly, tibialis anterior muscles from wt and mdx mice, both sedentary and exercised (long protocol), were homogenized in ice cold buffer containing 20 mM Tris-HCl (pH 7.4 at 4°C), 2% SDS, 5 mM EDTA, 5 mM EGTA, 1 mM DTT, 100 mM NaF, 2 mM sodium vanadate, 0.5 mM phenylmethylsulfonyl fluoride, 10 mg/mL leupeptin and 10 mL/mL pepstatin. Homogenates were centrifuged at 1500 x g for 5 min at 4°C. The supernatant obtained was quantified using Bradford protein assay kit (Bio-Rad Protein Assay Kit I5000001). 40 µg of protein was separated on a 10% SDS-PAGE and transferred on to nitrocellulose membranes for 1 h at 150 mA (SemiDry transferblot; Bio-Rad). Membranes were blocked for 2 h with Tris-HCl 0.2 M, NaCl 1.5 M, pH 7.4 buffer (TBS) containing 5% non-fat dry milk and 0.5% Tween-20, incubated overnight at 4°C with primary antibodies. The following dilution of primary antibodies were used: AMPK (rabbit polyclonal, Cell Signaling Technology) 1:1000, phosphorylated AMPK (rabbit polyclonal, Cell Signaling Technology) 1:1000 with TBS containing 5% non-fat dry milk. After three washes with TBS containing 0.5% tween-20 (TTBS), membranes were incubated for 1 h with secondary antibody labeled with peroxidase (1:5000 anti rabbit IgG, Sigma-Aldrich). Membrane was then washed with TTBS, developed with a chemiluminescent substrate (Clarity Western ECL Substrate, Bio-rad) and visualized on a Chemidoc imaging system (Bio-Rad). Densitometric analysis was performed using Image Lab software (Bio-rad). The software allows the chemiluminescence detection of each experimental protein band to obtain the absolute signal intensity. The density volume was automatically adjusted by subtracting the local background.

### **Determination of Matrix metalloproteinase-9 (MMP-9) level**

Blood was obtained from cardiac puncture of the left ventricle with a heparinized syringe and collected in heparinized tubes. Plasma samples were obtained after centrifugation for 20 minutes at 3700 rpm; then an additional centrifugation step at 11700 rpm for 10 minutes at 4 °C was performed for complete platelet removal. Matrix metalloproteinase-9 (MMP-9) was measured in 20-fold diluted plasma samples by enzyme-linked immunosorbent assay (Quantikine® ELISA Mouse Total MMP-9 Immunoassay R&D Biosystems ELISA), according to the manufacturer's instructions. The optical density of each well was determined, using a microplate readerset to 450 nm (Victor 3V, Perkin Elmer). A standard curve was generated to obtain MMP-9 levels expressed in ng/mL.

### **Muscle histology**

Six-eight µm transversally cut sections were stained with hematoxylin-eosin to calculate the percentage of both healthy myofibers with peripheral nuclei (peripherally nucleated fibers), regenerating/regenerated myofibers, showing central nuclei (centrally nucleated fibers) as well as the areas of damage (necrosis, inflammation and non-muscle areas). Morphometric analysis was performed by using Image J analysis software on the entire muscle section, randomly selecting about 3 fields/section at 10X for muscle damage and 6 field/section at 20X for counting centronucleated and peripherally nucleated myofibers. For each muscle, two sequential cross sections were used; at least five animals per experimental group were assessed (11).

### **Determination of transforming growth factor-β1 level (TGF-β1)**

Total TGF-β1 protein was measured by enzyme linked immunosorbent assay (ELISA), according to the manufacturer's instructions (RandD System, Minneapolis, MN, USA). Briefly, 10-20 mg of frozen muscle tissue was homogenized in 500 µL of a solution containing 1% Triton X-100, 20 mM Tris pH 8.0, 137 mM sodium chloride, 10% glycerol, 5 mM ethylenediamine tetraacetic acid and 1

mM phenyl methyl sulphonyl fluoride. TGF- $\beta$ 1 levels were expressed as pg of TGF- $\beta$ 1/ $\mu$ g of total protein content (11).

## **Statistics**

All experimental data were expressed as mean  $\pm$  standard error (SEM). Statistical analysis was performed to assess differences between genotypes (wt vs. mdx) or the effect of exercise within each genotype (sedentary vs. exercised). Multiple statistical comparisons between groups were performed by one-way ANOVA, with Bonferroni's t test post-hoc correction to provide a better evaluation of intra- and inter-group variability and avoid false positives. If necessary, statistical analysis for direct comparison between two means was performed by unpaired Student's t test.

## **RESULTS**

### **Effect of exercise on in vivo forelimb strength and torque**

The time-dependent effect of the treadmill exercise protocol on in vivo forelimb strength and body mass of mdx mice has been extensively described (5, 9, 11, 12). We used this treadmill protocol to assess if each of the experimental groups, but particularly group 2 (e.g., long protocol) showed the expected changes. As predicted, an age- and genotype-dependent increase in body mass was observed, with mdx being heavier than wt mice at T4, T8 and T12 (Fig.2A). We measured both the absolute (Fig.2B) and normalized (force/body mass; Fig.2C) forelimb strength. In agreement with previous studies (5, 12), a reduction in both absolute and normalized strength after 4 weeks of exercise was observed in the mdx animals. This impairment was maintained up to 12 weeks of exercise. Importantly, there was a significant increase in maximal forelimb strength of exercised wt mice vs. non-exercised counterparts at T8 and T12 suggesting a functional adaptation to training; however, this increase was not significant for the normalized values (Fig. 2B and C).

Grip strength determination may depend on mice volition, while torque measurements in anaesthetized mice are a pure measurement of neuromuscular function. Fig. 3 shows the tetanic torque measurements of wt and mdx mice at T4 and T12. Mdx mice with or without exercise, were significantly weaker than wt mice. However, no effect of exercise on the torque of mdx or wt mice was observed at T4 (Fig. 3A). Similar characteristics were observed at T8 (data not shown), and at T12 (Fig. 3B). Importantly, at this age the exercised mdx mice became significantly weaker than the sedentary ones, revealing a time-dependent effect of exercise on neuromuscular function of dystrophic phenotype.

### **Effect of exercise on contractile parameters of diaphragm and EDL muscles ex vivo**

#### *a) Isometric contraction in Diaphragm*

We assessed the effect of exercise on contractile performance of diaphragm, the mdx muscle that most resembles the progression of the human disease (49). In line with our previous data (11), DIA strips of mdx mice of both groups 1 and 2 showed a lower normalized twitch and tetanic tension with respect to those of wt mice (Fig. 4A and B). The chronic exercise protocol, either short or long, lead to a slight reduction of twitch and tetanic force of mdx DIA with respect to sedentary counterparts ranging between 10 and 20% (Fig. 4A and B), while no decrease in force was observed on wt diaphragm. No effects were observed in relation to genotype and exercise for kinetic parameters (data not shown). Another ex vivo functional parameter is fatigue. In group 1, mdx diaphragms were more fatigable than wt ones, with a greater and significant tension reduction of 15% vs. wt (force drop calculated at the 5<sup>th</sup> pulse;  $-2.3 \pm 1\%$  for wt mice, n=10, vs  $-7 \pm 1.4$  for mdx mice, n=11;  $p < 0.05$  by Student's t-test). A similar figure was observed for mice of group 2. The chronic exercise did not exert any effects on diaphragm fatigue in mdx mice (data not shown).

We then assessed the effects of a single bout of exercise on ex vivo contractions of diaphragm. Surprisingly, 24 hours after a single bout of exercise, we observed a significant reduction of normalized twitch (Fig. 5A) and tetanic tension (Fig. 5B) in mdx DIA with respect to the sedentary



counterparts. No significant changes in contraction kinetics nor in muscle fatigue were evident in response to the single exercise bout.

*b) Isometric contractions in Extensor digitorum longus muscles*

Fast-twitch muscles are mainly affected by dystrophic pathology, and hindlimb muscles directly contribute to running exercise (11, 21). We therefore focused on fast-twitch hind limb EDL muscle to evaluate the change in force generation in response to exercise. In line with other studies, the mass of the EDL muscle was greater in mdx compared to age-matched wt mice. The hypertrophy of mdx EDL muscle was observed in both group 1 and group 2 (Table 1). No effects of exercise were observed on mdx EDL muscles mass. However, in wt mice the muscle mass was significantly increased after the long exercise protocol, suggesting adaptive hypertrophy (Table 1).

Fig. 6 shows the significant decrease of twitch and tetanic tension of mdx EDL muscles vs. those of wt mice in both groups 1 and 2. Moreover, a time-dependent effect of exercise was observed on both twitch and tetanic forces of EDL muscles of mdx mice (Fig.6A and B, respectively). In particular, the contractile force of EDL muscles from exercised mdx mice of group 1 (short protocol) was reduced to about 10% with respect to that of sedentary mdx mice. This impairment became significant after the long protocol (Fig. 6A and B). No effect of exercise was observed on twitch and tetanic muscle force of wt mice after either short or long exercise protocols.

Kinetic parameters and fatigability of either wt or mdx EDL muscle were not modified by the exercise protocols in either group (data not shown).

We then assessed the effect of a single bout of exercise on EDL muscles. Interestingly, even after a single exercise protocol, both twitch and tetanic contractions of mdx EDL muscles were significantly lower with respect to those of sedentary counterparts (Fig.7A and B). Also, a single exercise bout decreased time-to-peak (TTP,  $14.4 \pm 0.6$  ms vs  $16.3 \pm 0.3$  ms,  $p < 0.001$ ) and half-relaxation time (HRT,  $10.8 \pm 0.3$  ms vs  $13.5 \pm 1.1$  ms) for exercised compared to non-exercised mdx mice, respectively. Fatigue was unchanged by a single exercise bout (data not shown).

*c) Eccentric contractions of EDL muscles*

Repeated lengthening (eccentric) contractions lead to a drop of isometric force, especially in more susceptible dystrophic muscles (57). Isolated EDL muscles were subjected to a protocol of 10 eccentric contractions; the expected decline in isometric force was observed in both groups (Fig. 8A and D). The force deficit was determined as the percent change in maximal isometric force between the 5<sup>th</sup> and the first eccentric contraction. In sedentary mice, as shown in Fig. 8B, this protocol led to a force drop of  $9\% \pm 1.6$  in mdx EDL muscles while a gain of  $1.9\% \pm 1$  was observed in wt muscles. Interestingly, the exercise exerted different effects in wt vs. mdx mice in group 1 (short protocol). Isometric force of EDL muscles of exercised mdx mice declined ( $-12\% \pm 3$ ). In contrast, exercised wt EDL muscles not only were resistant to eccentric contractions, but showed an increase in force at the 5<sup>th</sup> contraction by  $+13.3\% \pm 5$  (Fig. 8B). In the long exercise protocol (Fig. 8E), the exercised wt EDL muscles demonstrated resistance to eccentric contraction, although less than that for the short protocol, with an increase of force of  $0.2\% \pm 4$ , while sedentary wt mice showed a decreased force of  $7\% \pm 4$ . This beneficial adaptation was not observed in the mdx mice of group 2 as both sedentary and exercised mdx EDL muscles showed a remarkable force drop of about 20%. We then assessed the outcome of genotype and exercise on recovery from eccentric injury. Four minutes after the stretch contractions, the force of EDL muscle from sedentary wt mice of both groups recovered to 78% of the tetanic force obtained before the eccentric protocol, while recovery was complete after 30 minutes. The EDL muscles of age-matched mdx mice were more compromised by eccentric contractions, with force recovery markedly lower than that of wt; the recovery was also incomplete after 30 minutes, with a slightly worse profile in the mdx mice of group 2 vs group 1 (Fig. 8C and F). Recovery efficiency was influenced by exercise only in wt muscles. In particular, the latter showed an accelerated recovery for both the short and long exercise protocols. Accordingly, 30 minutes after the eccentric contractions, the EDL of exercised wt mice showed a greater force recovery than that of sedentary counterparts (Fig. 8C and F). By contrast, in

mdx EDL muscles no beneficial effects of exercise at either training duration were observed on recovery after 4 or 30 minutes (Fig. 8C and F).

We then assessed the outcome of a single bout of exercise. Again, 24 hours post-exercise, the EDL muscles from wt mice showed a significant increase of resistance to eccentric contractions compared to non-exercised ones, while the EDL muscles of mdx mice both sedentary and exercised, showed a similar tension reduction at the 5<sup>th</sup> eccentric contraction; both groups were significantly more affected than wt ones (Fig.9A and B). Again, the wt muscles efficiently recovered after 30 min; however, an efficient recovery was also found in acutely exercised mdx muscles (Fig. 9C).

### **Effect of long exercise protocol on gene expression**

Real-time PRC experiments were performed on contralateral EDL muscles of wt and mdx mice undergoing or not the long exercise protocol, in order to assess the expression of key genes known to be modulated by exercise, or to be involved in metabolic, vascular and structural remodeling.

#### *a) Exercise-related and myofiber phenotype genes*

As shown in Fig. 10, the chronic exercise protocol did not exert any significant adaptive changes in the expression of Pgc-1 $\alpha$  and sirtuin-1 (Sirt1), two molecules with a key role in metabolic adaptation to exercise, in wt EDL muscle. By contrast, the exercised mdx mice showed a significant down-regulation of Pgc-1 $\alpha$  and a marked decrease of the basal upregulation of Sirt1, corroborating the altered mechanical sensitivity and the insufficient metabolic adaptation of dystrophic muscle (9). In parallel, a decrease in Myosin Heavy Chain 1 (Myh1), as a marker of metabolic fiber-type remodeling, was observed in exercised mdx muscles, while no exercise-induced changes were observed in wt ones.

#### *b) Genes markers of angiogenesis and denervation*

Previous results highlighted an increased expression of vascular endothelial growth factor-A (Vegfa), a main inducer of angiogenesis, in GC mdx muscles with respect to wt ones, without any exercise-induced change in its expression (9). Considering that VEGFs are main target of hypoxia-

inducible factor-1 (HIF-1 $\alpha$ ), we have investigated the expression of Factor Inhibiting HIF-1 $\alpha$  (Fih1), to assess the long-term control of hypoxia pathways. Interestingly, the Fih1 expression was significantly decreased in mdx muscles vs wt, with no effects of exercise in either genotypes (Fig.10). This supports the maintenance of an hypoxia state in dystrophic muscle, underlying the enhanced VEGF signals. In parallel, we assessed the expression of Cd31, (platelet and endothelial cell adhesion molecule 1) as a marker of endothelial cells and of the efficiency of angiogenesis program. Interestingly, this marker was down-regulated in mdx muscles of either sedentary and exercised animals with respect to wt, suggesting an impairment of angiogenesis in dystrophic muscles, rather independent of physical activity. In order to assess the events that occur at the level of the neuromuscular junction, we measured the expression of Acetylcholine receptor subunit alpha1 (Achr1). We found a slight increase of the expression for Achr1 in mdx muscles with respect to wild-type mice. However, the long exercise protocol led to a 2-fold increase of Achr1 expression solely in mdx mice, which resulted significantly higher with respect to wt counterparts. This underlines an impairment of neuromuscular junction in dystrophin-deficient muscles which is exacerbated by the chronic exercise protocol.

*c) Muscle damage- and regeneration-related genes*

In line with previous observations in GC muscle, two genes markers of oxidative stress and cytoskeletal disorganization such as NADPH oxidase 2 (Nox2), and tubulin- $\alpha$ 1b (Tuba-1b), remained markedly upregulated in exercised mdx EDL muscle, while a marked decrease of the basal upregulation of follistatin (Fst), involved in regeneration, was observed. Again no changes in the above genes were observed in wt EDL muscle upon chronic exercise (Fig.10).

*d) Genes marker of muscle stiffness and cell-matrix interactions*

The genotype-related effects of exercise were also assessed on the expression of proteins of extracellular matrix involved in stiffness and fibrosis, such as collagene type I alpha 1 (Col1a1) and type III alpha 1 (Col3a1) and in elasticity, such as elastin (Eln). The expression of integrin  $\alpha$ 7 (Itga7) involved in the signaling between extracellular matrix and myofibers was also evaluated. As

shown in Table 2, no significant differences were observed in the various experimental conditions. However, and in line with data in the literature (24, 31), *Colla1* and *Itga7* were markedly increased of about 3- and 2-fold, respectively, in mdx muscles vs. wt ones, irrespective to the exercise protocol. A 2-fold increase was observed in *Eln* expression in exercised wild-type muscle vs sedentary counterparts; this could be indicative of a beneficial exercise-mediated adaptation to stretch of wt muscle, as occurring during eccentric contraction.

In order to better evaluate this latter point and considering that compliance to stretch is due to the relative contribution of elastic and static components, the ratio of *Eln* to *Colla1* and *Col3a1*, individually or in combination, were assessed (Fig.11 A, B and C). The ratio of *Eln* to *Colla1* plus *Col3a1* of exercised wt muscle was significantly increased with respect to wt muscle, while no significant changes were observed in dystrophic muscles (Fig.11A). This difference was more remarkable when the ratio *Eln* to *Colla1* was measured (Fig. 11B), while the ratio of *Eln* to *Col3a1* did not show significative changes (Fig.11C).

### **Effect of long exercise protocol on AMPK protein level**

AMP-activated protein kinase (AMPK) is a multisubstrate kinase working as main metabolic sensor, being activated in skeletal muscle during exercise and involved in PGC-1 $\alpha$  pathway (36, 50). Therefore, we assessed by Western blot the AMPK signaling by measuring the ratio of phosphorylated AMPK (pAMPK)/AMPK in TA muscle. The long exercise protocol led to a significative increase of pAMPK/AMPK in wt muscles with respect to sedentary counterpart, corroborating the exercise-induced activation of AMPK. The ratio pAMPK/AMPK of sedentary mdx muscles was higher with respect to that of wt ones, although not significantly. However, no significant change in pAMPK/AMPK was detected in the exercised dystrophic muscles (Fig.12).

### **Outcome of long exercise protocol on histopathology and biomarkers of muscle damage**

We assessed the effects of the long protocol of exercise on the histopathology of hind limb muscles of the different experimental groups. Representative images of gastrocnemius (GC) muscle sections of sedentary or exercised wt and mdx muscles are shown in Fig 13. Mdx muscles clearly demonstrate typical dystrophic features including alteration of muscle architecture, areas of necrosis, infiltrates, a large non-muscle area, likely due to the deposition of fibrotic and adipose tissue, and several centronucleated myofibers, an index of degeneration-regeneration (Fig. 13).

The typical histological features of dystrophic muscle were clearly detectable in the exercised group, while the exercise did not lead to any evident change in the muscular architecture of wt muscle. However, great variability between different mice and also within different fields of the same muscle were observed. A more detailed morphometric analysis on a large number of muscle samples per group was performed to assess possible quantitative differences. As shown in Fig.14A, the long protocol of chronic exercise in mdx muscles showed a 40% increase, although not statistical significant, in the area of total damage, considered as the sum of necrosis, infiltration and non-muscle area, versus that of sedentary counterparts, and no marked differences were observed in wt muscles. No differences were observed in the percentage of centronucleated myofibers between sedentary ( $61.4 \pm 4.2\%$ ) and exercised ( $63.3 \pm 1.5\%$ ) mdx muscles. To gain more insight into the possible increase in fibrotic signaling as a result of the long exercise protocol, the level of TGF- $\beta$ 1 protein, the major fibrotic cytokine, was measured using an enzyme-linked immunosorbent assay (ELISA). As expected, the level of TGF- $\beta$ 1 appeared higher in mdx vs. wt, but values were not different likely because of the high intergroup variability; no marked modification was observed as a consequence of exercise (Fig.14B).

Matrix metalloproteinases (MMP) constitute a family of zinc- and calcium-dependent endopeptidases that function in remodeling of extracellular matrix (ECM). The plasmatic levels of MMP-9 are considered a reliable biomarker of dystrophic pathology and an activator of latent TGF- $\beta$ 1 (40). Fig.14C shows an increase of plasma MMP-9 levels in mdx vs wt mice. There were no differences between the exercised and sedentary conditions within genotypes. However, the value

of MMP-9 value of exercised mdx muscle was significantly higher with respect to that of wt ones (ANOVA with Bonferroni post-hoc correction).

## DISCUSSION

The results of the present study disclose a complex equilibrium between adaptation and maladaptation of contractile properties of dystrophin-deficient muscle in response to chronic exercise, highlighting the involvement of both muscular and non muscular mechanisms. Dystrophin is considered to provide mechanical stability to the sarcolemma and to modulate mechano-transduction signaling via the DGC complex. Not surprisingly, muscles lacking dystrophin are particularly vulnerable to enforced muscle work. Dystrophic mdx mice, a model of DMD, show reduced strength and significant fatigue *in vivo* when subjected to a chronic non-eccentric treadmill exercise without effects in wildtype animals. Accordingly, this is a strategy for better discriminating the two genotypes, while *in vivo* outcomes measures are primary endpoints for longitudinal evaluation of pathology and drug effectiveness (5, 9, 11, 13, 14, 56).

However, *in vivo* limb force weakness in non-anesthetized mdx animals is a function of multiple neural and physiologic systems, including animal volition (12, 21). Our torque measurements in anesthetized animals disclosed, for the first time, that mdx mouse weakness is in fact related to a time-dependent exercise-induced impairment of neuromuscular function, while beneficial adaptation seems to occur in wt mice over time. The functional torque impairment in treadmill-exercised mdx mice mimics a more severe dystrophic condition, i.e. the severely affected golden retriever dystrophic dog (GRMD) model has hindlimb torque reduced by more than 50% with respect to healthy controls (52). Both muscular and nervous components can play a role in the damaging effect of exercise on torque force of mdx mice. Importantly, defects in neuromuscular transmission and neuromuscular junction (NMJ) morphology have been described in dystrophic mdx mice; it is likely that they are further aggravated with chronic exercise (2, 19, 43). In line with

this, we presently found an increased expression of cholinergic nicotinic receptor alpha 1 subunit, a clear marker of denervation-like process (32).

The assessment of the impact of chronic exercise on contractile properties of isolated dystrophic muscles was instead a strategy to estimate the occurrence of tissue-specific adaptation to mechanical challenge (8). In our *ex vivo* analyses, we focused on the contractile performance of two key muscles: the diaphragm, which is more compromised by pathology because of continuous respiratory activity, and the hind limb EDL muscle composed of type IIb fast-twitch glycolytic myofibers, which are more susceptible to dystrophic pathology and to contraction-induced injury compared to slow twitch myofibers (18, 42, 49).

Twitch and tetanic tension of both muscles are already significantly compromised by the pathology, allowing a clear discrimination between genotypes. We demonstrated, for the first time, that our chronic treadmill exercise protocol did not have overt effects in force output and kinetic in wt muscles, while producing a decrease of force of isolated mdx muscle; in particular the EDL muscles were significantly weakened by the long exercise protocol. Interestingly, a single bout of exercise induced a remarkable acute impairment of muscle contractile force in both mdx muscles. This is in line with other studies showing a marked effect of a single exercise bout on indices of muscle damage in dystrophic animals (46). Then, the mdx mouse muscles, and especially diaphragm, are acutely weakened by exercise; however they can functionally recover with an efficiency that is influenced by muscle type and by the amount of mechanical perturbation they face.

Adaptability appears to be compromised in the EDL muscle. In this regard, it was important to assess the effect of chronic exercise on the force drop in response to eccentric-contractions, as this approach could reveal an exercise-dependent susceptibility of dystrophic muscle to structural damage (57). As expected, the mdx compared to wt EDL muscle showed a marked force impairment during lengthening contractions. However, neither acute nor chronic treadmill exercise led to a greater impairment of mdx muscle during lengthening contractions. An intriguing finding, however, was that our treadmill exercise protocol led to a beneficial adaptation in wt but not mdx



muscles. Wt demonstrated greater resistance to loss of force during, and a faster recovery following eccentric contractions. Considering that eccentric contractions occur in vivo to slow or stop limb movements, such an adaptation toward lengthening contractions may be a protective adaptation in wt muscle against the potential for structural damage when exposed to repetitive physical activity (45). Importantly, our results show that this adaptation may result from an increased expression of elastin over collagens in exercised wt muscle; accordingly Gilbert et al., have recently described an increase of elastin level in trained healthy rats, mostly observed at protein level (20). This change may lead to increase compliance of muscle to stretch, while a failure in this adaptive process, along with the greater fibrosis, may, in part, account for the greater force drop in mdx mice after chronic in vivo exercise.

Impairment in structural remodeling can parallel and in part account for the altered signaling between mechanical activity and metabolic response. In line with this view, we confirmed in the fast-twitch EDL muscle an expression profile for key factors involved in mechanical-metabolic coupling and repair such as Sirt1, Pgc-1 $\alpha$  and follistatin (9, 22, 36), that differed by genotype and exercise condition. The selective down-regulation of protective and regeneration-related transcripts in response to exercise occurred only in mdx mice, in parallel with a lack of activation of AMPK signaling. These changes support the hypothesis that failing mechanical-metabolic coupling is the molecular mechanism reinforcing chronic damage, considering that Nox2 and tubulin- $\alpha$ 1b and other damage-related genes, remain up-regulated and poorly counteracted (9). Importantly, the enhanced expression of tubulin- $\alpha$ 1b is directly involved in cytoskeletal disorganization and in the mechanically induced oxidative stress in dystrophic myofibers and cardiomyocytes, and may also account for the increased calcium entry through mechano-sensitive channels after chronic exercise (1, 27, 44, 47). Nevertheless, although mdx muscles demonstrated a typical dystrophic histological profile and a greater plasma level of the biomarker MMP-9 compared to wt muscles, exercise slightly and not significantly exacerbated these alterations. However, it is clear that beneficial

functional adaptations to the treadmill exercise observed in wt muscles such as a force-gain vs a force-drop following eccentric contractions, were not evident in mdx muscles.

An additional mechanism underlying functional contractile impairment and weakness in chronically exercised mdx mice could be related to insufficient oxygen supply during contraction due to the occurrence of functional ischemia or to a defective microvasculature remodeling (41).

The role of vasculature impairment in dystrophic muscle damage is still controversial (34). Available evidence suggests there is a compensatory increase in microvasculature and arteriogenesis in young mdx mouse muscles (4, 51). However our gene expression study showed that the activation of pro-angiogenic signals, as supported by an increase in VEGFs (9) and by the reduced expression of Fih1, is not accompanied by an increased expression of markers of angiogenesis, such as Cd31. The insufficient angiogenesis may be directly related to missing additional actions of Fih1, i.e. this factor is required for vascular endothelium cell survival and is an oxygen sensor involved in metabolic homeostasis (28, 58). Although a more direct measure would be required to finally assess the lack of exercise-induced vascular remodeling, the overlapping gene expression in exercised and non exercised animals does not favour this mechanism in the exercise-induced weakness in vivo.

This latter can however derive from functional ischemia. In fact, it is well known that the lack of dystrophin and the disassembly of the DGC cause a delocalization of nNOS and impaired nitric oxide (NO) signaling. Coupled with altered shear stress in the vasculature, insufficient vasodilation during training activity can result (1, 29, 33, 35, 39). The above mechanism can also be aggravated in vivo by a defective sympatholytic reflex, especially during exercise (53). In addition, the NO-pathway, a primary defective signaling in dystrophin-deficient myofibers and representing a main therapeutic target in DMD (10), may also have an impact on the impairment of metabolic-mechanical coupling in dystrophic mouse muscle. In fact, NO/cGMP-dependent signaling is one of the multiple pathways converging on the activation of the co-transcription factor PGC-1 $\alpha$  (22, 55).

Finally, our results are the first evidence that the worsening conditions observed in the otherwise benign mdx phenotype with the use of treadmill exercise might be related to complex mechanisms involving defects at both muscular and non-muscular systems. A potential failure in the mechanical-metabolic coupling and related signals important for adaptation of muscle to mild exercise may add to defects of vascular and nervous system in response to exercise. Considering that mechanical challenge is a main inducer of muscle plasticity, our results showed a certain degree of adaptation of dystrophic muscle over chronic exercise, which is however not sufficient to overcome weakness, while being accompanied by the maintenance of a severe morphometric and transcriptional profile. Although clarification of the underlying mechanisms requires additional studies, the results provide some guidance for the development of effective treatments and proper exercise protocols for physical therapy of DMD patients.

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## **DISCLOSURES**

The authors disclose no conflict of interest

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## FIGURE CAPTIONS

### **Fig.1. Summary scheme of protocol timelines and endpoints for mice of groups 1 and 2**

The figure shows the protocol timelines for group 1 and 2 mice. At the beginning of the in vivo phase (T0), all mice of both groups were 4-5 weeks old. The dotted arrow indicates the period for which the exercised mice of both groups underwent a chronic treadmill exercise protocol. The exercised mice (wt and mdx) of group 1 were subjected to a “short protocol” of exercise (~4 weeks) while those of group 2 were subjected to a “long protocol” of exercise (> 20weeks). All animals of group 1 were exclusively used for ex vivo physiology measurements at the end of 4 weeks of exercise and/or cage-based activity. All mice of group 2 were monitored weekly for body mass (BM) and forelimb strength by grip test. These measurements were performed at the beginning (T0), and after 4 (T4), 8 (T8) and 12 (T12) weeks of either exercise or cage-based activity. At the same time points, the in vivo torque of all animals was also assessed. All mice of group 2 were used at the end of 20 weeks (T20) for ex vivo experiments: contraction measurements, ELISA assay, histology and qRT-PCR analysis.

### **Fig.2. Time-dependent effects of exercise on the in vivo parameters of wt and mdx mice**

This figure shows the variations in body mass (A), forelimb force (B) and forelimb force normalized to body mass (C) at the beginning (T0) and after 4 (T4), 8 (T8) and 12 (T12) weeks of

the treadmill-exercise protocol for wild-type (wt) and mdx mice. Parameters of non-exercised (sed) wt and mdx mice are also shown. All values are mean  $\pm$  S.E.M. from 8-10 mice for each group. A significant difference among groups was found by ANOVA analysis for A ( $F > 6$ ;  $p < 0.002$  for values from T4 to T12), B ( $F > 4.5$ ;  $p < 0.01$  for values from T0 to T12) and C ( $F > 12.6$ ;  $p < 1 \times 10^{-5}$  for values from T0 to T12). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \*sedentary wt mice ( $3 \times 10^{-8} < p < 0.004$ ), °exercised wt mice ( $3 \times 10^{-10} < p < 0.003$ ), #sedentary mdx mice ( $5 \times 10^{-6} < p < 0.002$ ).<sup>†</sup>An intra-genotype statistically significant difference between sedentary and exercised wt mice was found by unpaired Student's t test ( $p < 0.05$ ).

### **Fig.3. Time-dependent effects of exercise on in vivo tetanic torque of wt and mdx mice**

The figure shows isometric plantarflexor tetanic torque normalized to mouse body mass at increasing frequencies of stimulation after 4 (A, T4) and 12 (B, T12) weeks for both exercised or sedentary mice. Values are mean  $\pm$  S.E.M. from 6 mice per group. A significant difference among groups was found by ANOVA for A ( $F > 4.9$ ;  $p < 0.01$  for values at frequencies 30-100 Hz) and B ( $F > 9.03$ ;  $p < 0.0005$  for values at frequencies 1-200Hz). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \*sedentary wt mice ( $4.8 \times 10^{-5} < p < 0.008$ ), °exercised wt mice ( $8 \times 10^{-7} < p < 0.0005$ ). Statistical analysis between genotypes and within each genotype was also evaluated by Student's t-test; this showed a statistical difference for torque of exercised mdx mice versus sedentary counterparts at frequencies between 30 Hz and 180Hz ( $0.001 < p < 0.05$ ).

### **Fig.4. Time-dependent effects of exercise on ex vivo isometric contractile parameters of diaphragm of wt and mdx mice**

Shown are the normalized values of maximal isometric twitch (A, sPtw measured in kN/m<sup>2</sup>) and tetanic (B, sP0 measured in kN/m<sup>2</sup>) tensions of diaphragm strips from wt and mdx mice, that were exercised (short or long protocol) or not exercised (sedentary; sed). Each bar is the mean  $\pm$  S.E.M for 6-10 mice per group. A significant difference among groups was found by ANOVA analysis for A ( $F > 14.4$ ;  $p < 4 \times 10^{-6}$ ) and B ( $F > 15.4$ ;  $p < 2.6 \times 10^{-6}$ ). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \*sedentary wt mice ( $6.2 \times 10^{-5} < p < 0.001$ ) and °exercised wt mice ( $4.5 \times 10^{-6} < p < 0.0004$ ). The percentage variation of contractile forces between sedentary and exercised dystrophic mice, although not significant, is indicated above the dashed lines for a matter of clarity.

**Fig.5. Effects of acute exercise on ex vivo isometric contractile parameters of diaphragm muscle of wt and mdx mice**

Shown are the normalized values of maximal isometric twitch (A, sPtw measured in kN/m<sup>2</sup>) and tetanic (B, sP0 measured in kN/m<sup>2</sup>) tensions of diaphragm strips from wt and mdx mice. Data were collected from sedentary mice or from mice 24 hours after a single bout of exercise. Each bar is the mean  $\pm$  S.E.M. for 5-11 mice per group. A significant difference among groups was found by ANOVA analysis for A ( $F = 10.4$ ;  $p < 0.0002$ ) and B ( $F = 21$ ;  $p < 9 \times 10^{-7}$ ). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \*sedentary wt mice ( $2 \times 10^{-7} < p < 0.004$ ), °exercised wt mice ( $2 \times 10^{-5} < p < 0.006$ ) and #sedentary mdx mice ( $p < 0.006$ ). In the figure, is also shown the statistical significance by unpaired Student's t-test between genotypes or between sedentary and exercised animals of the same genotype.

**Fig.6. Time-dependent effects of exercise on ex vivo isometric contractile parameters of EDL muscle of wt and mdx mice**

Shown are the normalized values of maximal isometric twitch (A, sPtw measured in kN/m<sup>2</sup>) and tetanic (B, sP0 measured in kN/m<sup>2</sup>) tensions of EDL muscles from wt and mdx mice, exercised (short or long protocol) or not exercised (sedentary, sed). Each bar is the mean  $\pm$  S.E.M. for 8-14 mice per group. A significant difference among groups was found by ANOVA analysis for A ( $F > 8.6$ ;  $p < 0.0002$ ) and B ( $F > 3.5$ ;  $p < 0.02$ ). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \*sedentary wt mice ( $p < 2 \times 10^{-7}$ ), °exercised wt mice ( $p < 3 \times 10^{-8}$ ). In the figure is also shown the statistical significance by unpaired Student's t-test between genotypes or between sedentary and exercised animals of the same genotype.

**Fig.7 Effects of acute exercise on ex vivo isometric contractile parameters of EDL muscle of wt and mdx mice**

Shown are the normalized values of maximal isometric twitch (A, sPtw measured in kN/m<sup>2</sup>) and tetanic (B, sP0 measured in kN/m<sup>2</sup>) tensions of the EDL muscle from wt and mdx mice. Data were collected from sedentary mice or from mice 24 hours after a single bout of exercise. Each bar is the mean  $\pm$  S.E.M. for 5-11 mice per group. A significant difference among groups was found by ANOVA analysis for A ( $F = 19.31$ ;  $p < 1.4 \times 10^{-6}$ ) and B ( $F = 17.62$ ;  $p < 2.9 \times 10^{-6}$ ). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \*sedentary wt mice ( $3.5 \times 10^{-7} < p < 4.8 \times 10^{-5}$ ), °exercised wt mice ( $4 \times 10^{-5} < p < 0.005$ ) and #sedentary mdx ( $p < 0.01$ ). In the figure is also shown the statistical significance by unpaired Student's t-test between genotypes or between sedentary and exercised animals of the same genotype.

**Fig.8. Time-dependent effects of exercise on ex vivo force after eccentric contractions of EDL muscle of wt and mdx mice**

Shown are the change of isometric force produced during ten consecutive eccentric contractions of EDL muscles from wt and mdx mice, either sedentary (sed) or exercised with a short (A) or long (D) protocol. Each value is the percent variation with respect to the force at the beginning of the stimulation (first pulse) and is the mean  $\pm$ S.E.M. for 7-12 mice per group. In (B) and (E), the reduction of tensions calculated at the 5<sup>th</sup> pulse (see arrows in A and D) are shown. A significant difference among groups was found by ANOVA analysis for B ( $F=13.7$ ,  $p < 2.5 \times 10^{-6}$ ) and E ( $F=5.2$ ,  $p < 0.006$ ). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \*sedentary wt ( $p < 0.003$ ) and °exercised wt mice ( $9 \times 10^{-7} < p < 0.004$ ). In the figure is also shown the statistical significance by unpaired Student's t-test between genotypes or between sedentary and exercised animals of the same genotype.

The ability of wt and mdx muscles to recover from the drop in force at 4 and 30 minutes following the eccentric contractions is shown for the mice in the short (C) or long (F) exercise protocol. A significant difference among groups was found by ANOVA analysis for C ( $F > 8.2$ ,  $p < 0.0002$ , after 4 min) and for F ( $F > 31.4$ ,  $p < 6 \times 10^{-9}$ , after 4 and 30 min). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \*sedentary wt mice ( $2.8 \times 10^{-8} < p < 0.01$ ) and °exercised wt mice ( $4 \times 10^{-11} < p < 0.0002$ ). In the figure is also shown the statistical significance by unpaired Student's t-test between genotypes or between sedentary and exercised animals of the same genotype.

**Fig.9. Effects of acute exercise on ex vivo force after eccentric stimulation of EDL muscle of wt and mdx mice**

Shown is the change of isometric force produced during ten consecutive eccentric contractions of EDL muscles from wt and mdx mice. Data were collected from muscles of sedentary mice or from mice 24 hours after a single bout of exercise. Each value is the percent variation with respect to the force at the beginning of the stimulation (first pulse) and is the mean  $\pm$ S.E.M. of 5-7 animals for group. In B) is shown the reduction of tension calculated at the 5<sup>th</sup> pulse (see arrows in A). In C) is

shown the ability of wt and mdx muscle groups to recovery from eccentric contraction force drop 4 and 30 minutes after the end of the eccentric contraction train. A significant difference among groups was found by ANOVA analysis for B ( $F= 10$ ,  $p < 0.0003$ ) and C ( $F= 13.6$ ,  $p < 5.6 \times 10^{-5}$  for recovery after 4 minutes). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \*sedentary wt mice ( $9.9 \times 10^{-5} < p < 0.002$ ) and °exercised wt mice ( $6 \times 10^{-5} < p < 0.001$ ). In the figure is also shown the statistical significance by unpaired Student's t-test between genotypes or between sedentary and exercised animals of the same genotype.

**Fig.10. Effects of long protocol of exercise on expression of genes markers of metabolic adaptation or of vascular and neuromuscular junction remodeling in EDL muscles of wt and mdx mice**

The figure shows the effect of the long exercise protocol on gene expression in EDL muscle of wt and mdx mice. Transcript levels were determined by qPCR for Pgc-1 $\alpha$ , Sirt1, Myh1, Cd31, Fih1, Achr-1, Fst, Tuba-1b and Nox2 and normalized to the Gapdh transcript level. Each bar is the mean  $\pm$  S.E.M. of 3-7 mice, indicated in brackets. A significant difference among groups was found by ANOVA analysis for Pgc-1 $\alpha$  ( $F= 10.1$ ,  $p < 0.0008$ ), Sirt1 ( $F= 10.5$ ,  $p < 0.0007$ ), Cd31 ( $F= 9.2$ ,  $p < 0.0005$ ), Fih1 ( $F=11.8$ ,  $p < 0.0001$ ), Achr-1 ( $F= 4.1$ ,  $p < 0.02$ ), Fst ( $F= 6.4$ ,  $p < 0.007$ ) and Tuba-1b ( $F= 14.4$ ,  $p < 8.3 \times 10^{-5}$ ). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \* sedentary wt ( $4 \times 10^{-5} < p < 0.02$ ), °exercised wt mice ( $5 \times 10^{-5} < p < 0.05$ ) and #sedentary mdx mice ( $0.001 < p < 0.003$ ).

**Fig.11. Effects of long exercise protocol on expression of genes involved in muscle stiffness and elasticity in EDL muscles of wt and mdx mice**

The figure shows the effect of the long exercise protocol on muscle stiffness of wt and mdx mouse. In A) is shown the ratio of transcript levels (normalized to Gapdh), determined by qPCR, of elastin



(Eln) to both collagen type I alpha 1 (col1a1) and collagen type III alpha 1 (col3a1), in B) the ratio of elastin to Col1a1 and in C) the ratio of elastin to Col3a1. Each bar is the mean  $\pm$  S.E.M. of 4-6 mice, indicated in brackets. A significant difference among groups was found by ANOVA analysis for ratio of elastin with Col1a1 (Fig.11 B;  $F=6$ ,  $p < 0.007$ ). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \*sedentary wt ( $p < 0.04$ ) and °exercised wt mice ( $0.01 < p < 0.02$ ). In the figure 11A is also shown the statistical significance by unpaired Student's t-test between genotypes or between sedentary and exercised animals of the same genotype.

**Fig.12. Effects of long protocol of exercise on ratio of phosphorylated AMPK/AMPK in tibialis anterior muscles of wt and mdx mice**

Representative Western blots of the phosphorylated form of AMPK (p-AMPK) and total AMPK, (A). In B) is shown the ratio pAMPK / AMPK in tibialis anterior muscle from wild-type and mdx mice, both sedentary and exercised. Each bar is the mean  $\pm$  S.E.M. of 6-7 mice, indicated in brackets. A significant difference among groups was found by ANOVA analysis for ratio pAMPK/AMPK ( $F=3.72$ ;  $p < 0.03$ ). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \*sedentary wt ( $p < 0.04$ ). In the figure 12 B is also shown the statistical significance by unpaired Student's t-test between genotypes or between sedentary and exercised animals of the same genotype.

**Fig.13. Effects of long exercise protocol on the histological profile of wt and mdx mouse muscles**

Samples of haematoxylin-eosin staining showing the morphological profiles of gastrocnemious (GC) muscles from wt and mdx mice that were either sedentary or exercised. For a qualitative comparison, representative profiles of sedentary and exercised wt GC muscles are shown in the upper panels, while GC muscle sections from exercised and sedentary mdx mice are shown in the

lower panels. The latter clearly show a non-homogeneous pattern typical of dystrophic muscle, with great variability in fiber dimension, large areas of necrosis accompanied by mononuclear infiltrates and/or small regenerating fibers. The areas of non-muscle tissue are also visible. The images are at 10X magnification.

**Fig.14. Effects of long exercise protocol on total damaged muscle area, TGF- $\beta$ 1 as a marker of fibrosis and on plasma level of metalloproteinase-9 in wt and mdx mice**

In (A), the bars show the percentage of total damaged muscle area of GC muscle of wt and mdx mice that were either sedentary or exercised. Each bar is the mean of at least 5 muscles and 3 fields per muscle. A significant difference among groups was found by ANOVA analysis ( $F= 12.6$ ;  $p < 3.4 \times 10^{-5}$ ). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \*sedentary wt mice ( $2.5 \times 10^{-5} < p < 0.001$ ); °exercised wt mice ( $10^{-5} < p < 0.006$ ). The percentage variation of total damaged muscle area between sedentary and exercised mdx mice is indicated above the line. In (B), the bars show the levels of total TGF- $\beta$ 1 in gastrocnemious muscle, in both sedentary and exercised wt and mdx mice. Each bar is the mean  $\pm$  S.E.M. from 3-4 samples per group. A significant difference among groups was found by ANOVA analysis ( $F= 3.8$ ;  $p < 0.04$ ). Bonferroni post hoc correction did not reveal significant differences between means.

In (C), the histograms show the plasma levels of metalloproteinase-9 (MMP-9) of sedentary and exercised wt and mdx mice. Each bar is the mean  $\pm$  S.E.M. for 4-10 mice per group. A significant difference among groups was found by ANOVA analysis ( $F= 8.45$ ,  $p < 0.0005$ ). Bonferroni post hoc corrections for individual differences between groups are as follows: significantly different with respect to \*sedentary wt mice ( $p < 0.0005$ ) and °exercised wt mice ( $p < 0.0004$ ). Intra-genotype significant differences found by unpaired Student's t test are indicated above the bars. The percentage variation of MMP-9 plasma level of dystrophic mice is indicated above the dashed lines.

**Table 1. Effects of chronic exercise protocols on mass of EDL muscles of wt and mdx mice**

The table shows the values of EDL mass (mg) of mice groups subjected to the short (Group 1) or long (Group 2) exercise protocols, on not exercised (sed). A significant difference among groups was found by ANOVA analysis ( $F > 12.4$ ;  $p < 8.5 \times 10^{-6}$ ). Bonferroni post hoc for individual differences between groups are as follows: significantly different with respect to \*sedentary wt mice ( $7.7 \times 10^{-8} < p < 0.004$ ); °exercised wt mice ( $6 \times 10^{-6} < p < 0.0002$ ). §An intra-genotype statistically significant difference between sedentary and exercised mdx mice was found by unpaired Student's t test ( $p < 0.05$ ). †An intra-genotype statistically significant difference between sedentary and exercised wt mice was found by unpaired Student's t test ( $p < 0.05$ ).

**Table 2. Effects of long protocol of exercise on expression of genes involved in muscle stiffness and cell-matrix interactions in EDL muscles of wt and mdx mice.**

The table shows the values of transcript levels, determined by qPCR, for Elastin (Eln), Collagen type I alpha1 (Col1a1), Collagen type III alpha 1 (Col3a1) and Integrin 7 (Itga7) and normalized to the Gapdh transcript level. Each values is the mean  $\pm$  S.E.M. of 4-7 mice for group. No significant difference among groups was found by ANOVA analysis and Bonferroni post hoc correction.