

## RESEARCH

# Testosterone improves muscle fiber asset and exercise performance in a metabolic syndrome model

Erica Sarchielli<sup>1</sup>, Paolo Comeglio<sup>2</sup>, Sandra Filippi<sup>3</sup>, Ilaria Cellai<sup>2</sup>, Giulia Guarnieri<sup>1</sup>, Daniele Guasti<sup>1</sup>, Elena Rapizzi<sup>2</sup>, Giulia Rastrelli<sup>2</sup>, Daniele Bani<sup>1</sup>, Gabriella Vannelli<sup>1</sup>, Linda Vignozzi<sup>2,4,5</sup>, Annamaria Morelli<sup>1</sup> and Mario Maggi<sup>2,4,6</sup>

<sup>1</sup>Anatomy and Histology Unit, Department of Experimental and Clinical Medicine, University of Florence, Florence, Italy

<sup>2</sup>Sexual Medicine and Andrology Unit, Department of Biomedical, Experimental and Clinical Sciences 'Mario Serio', University of Florence, Florence, Italy

<sup>3</sup>Interdepartmental Laboratory of Functional and Cellular Pharmacology of Reproduction, Department of Neuroscience, Drug Research and Child Care, University of Florence, Florence, Italy

<sup>4</sup>I.N.B.B. (Istituto Nazionale Biostrutture e Biosistemi), Rome, Italy

<sup>5</sup>Andrology, Women's Endocrinology and Gender Incongruence, Careggi Hospital, Florence, Italy

<sup>6</sup>Endocrinology, Careggi Hospital, Florence, Italy

Correspondence should be addressed to A Morelli: [a.morelli@unifi.it](mailto:a.morelli@unifi.it)

## Abstract

Lifestyle modifications, including physical exercise (PhyEx), are well-known treatments for metabolic syndrome (MetS), a cluster of metabolic and cardiovascular risk factors often associated to hypogonadism. Given the trophic role of testosterone on skeletal muscle (SkM), this study was aimed at evaluating the effects of testosterone treatment on SkM metabolism and exercise performance in male rabbits with high-fat diet (HFD)-induced MetS. HFD rabbits, treated or not with testosterone (30 mg/kg/week) for 12 weeks, were compared to regular diet animals (RD). A subset of each group was exercise-trained for 12 weeks. HFD increased type-II (fast, glycolytic) and decreased type-I (slow, oxidative) muscle fibers compared to RD as evaluated by RT-PCR and histochemistry. Testosterone reverted these effects, also inducing the expression of mitochondrial respiration enzymes and normalizing HFD-induced mitochondrial cristae reduction. Moreover, testosterone significantly increased the expression of myogenic/differentiation markers and genes related to glucidic/lipid metabolism. At the end of the PhyEx protocol, when compared to RD, HFD rabbits showed a significant reduction of running distance and running time, while testosterone counteracted this effect, also decreasing lactate production. In the trained groups, muscle histology showed a significant reduction of oxidative fibers in HFD compared to RD and the positive effect of testosterone in maintaining oxidative metabolism, as also demonstrated by analyzing mitochondrial ultrastructure, succinate dehydrogenase activity and ATP production. Our results indicate that testosterone could be useful to promote oxidative muscle metabolism altered by MetS, thus improving exercise performance. Conversely, testosterone administration to otherwise eugonadal rabbits (RD) only increased muscle fiber diameter but not endurance performance.

## Key Words

- ▶ testosterone treatment
- ▶ muscle metabolism
- ▶ muscle fibers
- ▶ physical activity
- ▶ metabolic syndrome

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## Introduction

Metabolic syndrome (MetS) is a clustering of different metabolic alterations and cardiovascular risk factors, with insulin resistance being one of the main pathogenic factors. In men, MetS is also associated with a sex hormone imbalance characterized by low testosterone and gonadotropin levels, leading to hypogonadotropic hypogonadism (HH). The mechanism(s) underlying this association are complex and not fully elucidated. In fact, hormonal imbalance (low testosterone, increased estrogen/testosterone ratio) may be a consequence of metabolic dysregulation, but the converse may also apply with MetS being a consequence of testosterone deficiency (Morelli *et al.* 2016). Hence, the direction of this association is still undetermined.

The etiology of MetS is multifactorial and comprises both genetic and lifestyle factors (Morelli *et al.* 2016, Cangiano *et al.* 2019). The prevalence of MetS is increasing in all age/ethnic groups worldwide, primarily due to modifiable factors such as increased sedentary screen time, physical inactivity and diet (Roberts & Barnard 2005). Lifestyle modifications, including both dietary and physical training interventions, are well-known treatments for MetS. Exercise and physical activity are recommended for either the primary management of MetS individual risk factors (Pedersen & Saltin 2015) or for the prevention of MetS itself (Lakka & Laaksonen 2007). Moreover, physical activity levels and cardiorespiratory fitness display notable inverse relationships with MetS prevalence (Church 2011, Ingle *et al.* 2017). In particular, a recent review highlighted that aerobic exercise, conducted for a minimum duration of 12 weeks (three times/week), is beneficial for individuals with MetS without diabetes, with improvements in several cardiovascular risk factors (Wewege *et al.* 2018).

Skeletal muscle is the most abundant tissue in humans and represents 40–60% of total body weight in normal women and men, with a variety of physiological functions. Muscle loss results, therefore, in both physical alterations and metabolic derangements (Abrigo *et al.* 2016). Since skeletal muscle accounts for 80% of insulin-stimulated uptake of glucose, it represents one of the principal tissues for evaluating mechanism(s) related to insulin resistance (Stuart *et al.* 2013).

Muscles have a heterogeneous composition with a mosaic of different fiber types with peculiar physiological contractile properties. Skeletal muscle fibers are primarily classified as ‘slow-twitch’ (type I) and ‘fast-twitch’ (type II).

Moreover, based on differential myosin heavy chain (MYH) gene expression, there is a further classification of fast-twitch fibers into three major subtypes (types IIA, IIX, and IIB). Type I fibers primarily use oxidative metabolism and are more resistant to fatigue, while type II fibers partially or completely rely upon glycolytic metabolism ranging from IIA>IIX>IIB (Schiaffino & Reggiani 2011). Muscle is a versatile tissue and is capable of changing phenotypic properties in response to hormonal, lifestyle and environmental cues. Muscle metabolism and performance depend upon muscle plasticity and, therefore, upon the relative proportion of the fiber types. For example, it was demonstrated that the proportion of slow fibers is significantly increased in response to exercise training (Pette 2002).

Skeletal muscle mass is mainly regulated by exercise training, nutrition and hormonal stimuli that control anabolic-catabolic balance, glucose metabolism, muscle mass maintenance and reparation after injury. It is well known that sex steroids, in particular androgens, promote the growth/maintenance of muscle mass and strength and exert beneficial effects on metabolism and on fiber repair after injury (Carson & Manolagas 2015). Thus, physiologic or pathologic changes in the levels of circulating sex steroids have important implications for muscle health in both genders (Carson & Manolagas 2015). Healthy hypogonadal men and individuals with androgen deficiency (as in androgen deprivation therapy for prostate cancer or administration of GnRH agonists) have lower muscle size, strength and function, higher fat mass and higher incidence of insulin resistance, when compared to age-matched eugonadal men (Grinspoon *et al.* 1996, Bhasin *et al.* 2004, Gonzalez *et al.* 2016). Moreover, some studies demonstrated a correlation between the age-associated decline in serum testosterone levels and the reduced mass and strength in male lower limbs (Morley *et al.* 1997, Baumgartner *et al.* 1999, Roy *et al.* 2002). On the contrary, a dose-dependent increase in fat-free mass, muscle size, muscle protein synthesis and maximal voluntary strength was observed both in healthy androgen-deficient (Finkelstein *et al.* 2013) and in eugonadal men treated with graded doses of testosterone (Bhasin *et al.* 2004). Accordingly, a recent meta-analysis of randomized controlled trials (RCTs) employing testosterone treatment (TTh) and reporting data on body composition indicates that TTh is able to increase muscle mass and, as a consequence, glucose metabolism (Corona *et al.* 2016). In particular, TTh determines muscle fiber hypertrophy (i.e. increase in muscle fiber cross-sectional

area), but not hyperplasia, with an increase in satellite cell and in myonuclear number (Sinha-Hikim *et al.* 2002).

In MetS subjects, it has been demonstrated that skeletal muscles had a different arrangement of fibers respect to healthy subjects, with a significant reduction of type I fibers and a resulting muscle composition similar to that of patients with type 2 diabetes (Stuart *et al.* 2013). In addition, individuals with higher type I fiber content have a reduced risk for developing MetS and diabetes (Hernandez *et al.* 2001).

Animals fed a high-fat diet (HFD) show an impairment of muscle function and, specifically, the development of the typical features of muscle wasting (such as weakness, loss of muscle mass, decreased fiber diameter) and atrophy (Bhatt *et al.* 2006, Sishi *et al.* 2011). In HFD male mice, a decline in type I fibers and an increase in type I/IIA hybrid fibers were observed, suggesting that the type I fibers were transformed primarily into these hybrids and are most susceptible to the effects of obesity (Denies *et al.* 2014).

During the last 10 years, we set up a non-genetic model of MetS by feeding male rabbits with a high-fat, cholesterol-rich, diet (HFD). The resulting phenotype showed typical features of the human MetS (increased visceral obesity, hypertension, dyslipidemia, glucose intolerance), including hypogonadotropic hypogonadism, with a significant reduction of plasma testosterone and gonadotropins (FSH, LH), along with a decrease in testis and seminal vesicle weights (Filippi *et al.* 2009). In this experimental model, sex hormone imbalance was related to MetS severity, since testosterone decreased and E increased as a function of the number of MetS components (Morelli *et al.* 2016). Interestingly, we recently demonstrated that, when HFD rabbits were trained with aerobic physical activity on a treadmill, they exhibited a reversal of the hypogonadotropic condition, including a restoration of the hypothalamic network controlling GnRH production. However, these animals also showed a deficient muscular response to fatigue, as demonstrated by a significant reduction of both running distance and running time in comparison to the control group fed a regular diet (Morelli *et al.* 2019). Hence, they are less prone to perform physical exercise. Given the well-known trophic role of testosterone on skeletal muscle (SkM) mass, in this study, we extended the previous findings with the aim of analysing the effects of HFD on SkM composition and metabolism, along with the possible beneficial role of testosterone in preventing these effects and in improving exercise performance in the HFD-induced MetS model.

## Materials and methods

### Animals' treatment

Adult male New Zealand White rabbits (Charles River), weighing about 3 kg (15 weeks old), were individually caged under standard conditions in a temperature and humidity controlled room on a 12 h light:12 h darkness cycle. Water and food were unrestricted throughout the study and food intake was daily measured. After 1 week of regular diet (Harlan-Global Diet 2030, produced by Mucedola s.r.l, Milan, Italy; water 12%, protein 16.5%, fiber 15.5%, ash 8.5% and vegetable-derived fat 3.5%, total calories 3750 kcal/kg), animals were randomly divided into three groups: (1) control rabbits continued to receive a regular diet (RD group;  $n=25$ ); (2) treatment rabbits received a high-fat diet (HFD) enriched by 0.5% animal-derived cholesterol and 4% peanut oil, total calories 4000 kcal/kg (HFD group;  $n=27$ ), (3) HFD rabbits supplemented with a pharmacological dose of testosterone (30 mg/kg weekly i.m.; HFD+testosterone group;  $n=25$ ), as previously described (Filippi *et al.* 2009). To evaluate the effect of testosterone in control animals, an additional group of RD rabbits was supplemented with a pharmacological dose of testosterone (30 mg/kg weekly i.m.; RD+testosterone group,  $n=8$ ) for 12 weeks. A subset of rabbits for each group (RD,  $n=10$ ; HFD,  $n=12$ ; HFD+testosterone,  $n=10$ ; RD+testosterone  $n=4$ ) was trained with a physical exercise (PhyEx) running protocol on a treadmill with increasing intensity, duration and frequency during the 12 weeks, as previously published (Morelli *et al.* 2019) and described below. The remaining animals were left in the cages for the same period. The diet and training began at the same time and lasted 12 weeks. PhyEx rabbits were killed 90 min after the end of the exercise. Blood samples for glucose, total cholesterol, triglycerides, LH and testosterone measurements were collected as previously described (Morelli *et al.* 2019). In particular, for the PhyEx rabbits, blood samples were obtained immediately before (pre-exercise), immediately after (end exercise) and 90 min after the end (recovery) of the last exercise at week 12 for measuring blood lactate concentration, testosterone, LH, glucose and triglycerides. Mean arterial blood pressure (MAP) was measured and afterward, the rabbits were killed by a lethal dose of pentobarbital (Morelli *et al.* 2019). After killing, the vastus medialis muscle was rapidly dissected and appropriately stored for the subsequent analyses. To evaluate the prevalence of MetS, we previously designed an algorithm taking into account the presence, as a dummy variable,

of one or more of the following factors: hyperglycemia, high triglycerides, high cholesterol, increased blood pressure, and visceral fat accumulation. Cutoffs for each factor were derived by the mean  $\pm$  two s.d. of the analyzed parameter, as measured in a large database concerning RD rabbits ( $n=96$ ). Positivity for three or more factors identifies MetS (Maneschi *et al.* 2012). Animal handling complied with the Institutional Animal Care and Use Committee of the University of Florence, Florence, Italy, which approved the study, in accordance with Italian Ministerial Law, n. 326/2013-B.

### Physical exercise protocol

The physical exercise protocol consisted of a 12-week period of running on a treadmill designed to accommodate rabbits and produced by AL.TO. (Cavezzo, Modena, Italy). During the first 2 weeks, the rabbits ran 2 days/week at a treadmill speed of 10 m/min for 3 min with no inclination (0%). From the third week of treatment, the running speed and period were gradually increased with caution: in the third week, the rabbits ran for 5 min at 10 m/min continuously (2 days/week) with inclination (20%). During subsequent weeks, either the running velocity was extended 5 m/min each week until they could run at a speed of 20 m/min (from week 6) and the running time was extended 5 min each week until they could run for 25 min per day or until exhausted, with inclination (20%). From week 4, they were trained for 3 days/week for a total of 9 weeks. Exhaustion was objectively determined when the rabbit refused to run any longer and typically lay down and stretched its hindlimbs. During the exercise, animals were constantly monitored, and when they stopped running, the experiment was immediately considered concluded and the animals were moved to their cages under standard conditions. No adverse events from the exercise were registered.

### Plasmatic levels of cholesterol, triglycerides, glucose, testosterone, LH, and lactate

Plasma cholesterol, triglycerides and glucose levels were measured by using an automated system (ADVIA 2004 Siemens Chemistry System; Siemens Science Medical Solution Diagnostic), as previously described (Filippi *et al.* 2009). Plasma testosterone and LH levels were measured using a chemiluminescence system (Immunolite 2000 Siemens, Siemens Healthcare Diagnostics) and an ELISA kit (Repropharm Vet, Nouzilly, France), respectively, as previously described (Morelli *et al.* 2019).

Lactate was measured using an automated system according to the manufacturer's instructions (Lactate Scout, EKF Diagnostic-SensLab, Leipzig, Germany).

### Oral glucose tolerance test

Oral glucose tolerance test (OGTT) was performed as previously published (Filippi *et al.* 2009). Briefly, after an overnight fasting, a glucose solution (1.5 g/kg) was orally administered to the animals and blood samples were collected before and 15, 30, and 120 min after glucose loading for plasma glucose measurement. The incremental area under the curve (iAUC) was calculated using GraphPad Prism software, version 5.0 for Windows.

### RNA extraction and quantitative RT-PCR analysis

Isolation of total RNA from frozen muscles and RT-PCR analysis were performed as previously described (Morelli *et al.* 2019). Briefly, for isolation a TRIzol reagent (Life Technologies) and RNeasy Mini Kit (Qiagen) were utilized. cDNA synthesis was carried out using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). Quantitative real-time RT-PCR (qRT-PCR) amplification and detection were carried out using SsoAdvanced™ Universal SYBR® Supermix and a CFX96 Two-Color Real-Time PCR Detection System (Bio-Rad Laboratories). Specific PCR primers for rabbit target genes were designed based on sequences available at NCBI GenBank (<https://www.ncbi.nlm.nih.gov/genbank/>) or Ensemble Genome (<http://www.ensembl.org>). The 18S rRNA subunit was used as the reference gene for the relative quantification of the target genes based on the comparative threshold cycle (Ct)  $2^{-\Delta\Delta Ct}$  method.

### Histomorphometric analysis, fiber typing and Oil Red O staining

Some blocks of vastus medialis muscle were embedded in paraffin and used for morphometric evaluation and periodic acid–Schiff (PAS) staining. In detail, deparaffinized and rehydrated 5  $\mu$ m thick cross-sections were processed using standard hematoxylin-eosin staining, and fiber diameter was calculated using Image J 1.51f software (National Institutes of Health) in ten random images from at least three animals for each experimental group. Additional sections were stained with PAS (Bio-Optica S.p.A., Milan, Italy) according to the manufacturer's instructions. Light, medium and dark PAS stained fibers



were counted in ten random images from at least three animals for each experimental group.

Immediately after collection, other tissue blocks from the muscle belly were affixed to a cork disc with OCT support medium (Sakura Fintek Europe, Zoeterwoude, Netherlands), then frozen by immersion in isopentane, kept at the freezing point in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$ . Transverse muscle cryostat sections (8- $\mu\text{m}$  thick) were stained with a SDH assay or Oil Red O kit, following the manufacturer's instructions (Bio-Optica S.p.A.). The number of SDH positive fibers was counted in ten random images from at least three animals for each experimental group.

### Immunohistochemistry

Deparaffinized and rehydrated 5- $\mu\text{m}$ -thick sections of vastus medialis muscle were processed using immunohistochemical procedures. Briefly, sections were incubated overnight at  $4^{\circ}\text{C}$  with mouse monoclonal anti-PAX-7 primary antibody (1:50 dilution; sc-81975, Santa Cruz Biotechnology) or anti-MYH3 primary antibody (1:30 dilution; sc-53091, Santa Cruz Biotechnology) and subsequently with biotinylated anti-mouse (Thermo Scientific) followed by streptavidin-peroxidase complex (Thermo Scientific). The reaction product was developed with 3',3'-diaminobenzidine tetrahydrochloride as chromogen (Sigma-Aldrich). Negative controls were performed avoiding primary antibody. Slides were evaluated and photographed using a Nikon Microphot-FXA microscope. The number of PAX-7 positive cells was counted in ten fields from three animals for each group and expressed as a percentage of the RD group (mean  $\pm$  S.E.M.).

### Electron microscopy

Skeletal muscle specimens were fixed in 4% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 24 h, post-fixed in 1% osmium tetroxide in 0.1 M phosphate buffer, pH 7.4, for 1 h, dehydrated in graded acetone and embedded in Epon 812 epoxy resin (Fluka, Buchs, Switzerland). Ultrathin sections were stained with uranyl acetate and alkaline bismuth sub nitrate and examined under a JEM 1010 electron microscope (JEOL) at 80 kV. Micrographs were taken at  $\times 50,000$  final magnification using a Mega View III digital camera (SIS-Soft Imaging System, Munster, Germany) in at least ten fields for each sample. Mitochondrial and internal cristae surface area was measured using iTEM image analysis software (SIS-Soft).

### Triglycerides content

Triglycerides content from skeletal muscle biopsies in six animals from each experimental group was evaluated using the Triglyceride Quantification Colorimetric/Fluorometric Kit (BioVision), following the manufacturer's instructions. Briefly, 100 mg of frozen tissue was mechanically homogenized and resuspended in 1 mL solution containing 5% NP-40 in water, heated to  $80\text{--}100^{\circ}\text{C}$  to solubilize all triglycerides and centrifuged at top speed for 2 min. Lipase was subsequently added and incubated for 20 min at room temperature to convert triglycerides to glycerol and fatty acids. After the addition of Triglyceride Reaction Mix, absorbance at 570 nm was read with a plate reader (Victor3 1420, Perkin Elmer).

### Tissue homogenates and lysates preparation

Homogenates were prepared, and succinate dehydrogenase (SDH) activity was measured as previously described (see below and [Rapizzi \*et al.\* 2015](#)). Small frozen skeletal muscle sections were first chopped finely, and samples were then homogenized in a hand-held plastic homogenizer (80 strokes), in a solution containing 120 mM KCl, 20 mM HEPES, 2 mM  $\text{MgCl}_2$ , 1 mM EGTA and 5 mg/mL BSA. Homogenates were centrifuged at  $800\text{ g}$  for 10 min at  $4^{\circ}\text{C}$ , and the enzymatic assay was performed on the supernatant. For lysates, pieces of finely chopped skeletal muscle were lysed in buffer containing 50 mM Tris-HCl pH=7.5, 120 mM NaCl, 1 mM EGTA, 6 mM EDTA, 15 mM  $\text{Na}_4\text{P}_2\text{O}_7$ , 20 mM NaF and 1% Triton X-100 protease inhibitor cocktail. Lysates were clarified by centrifugation at  $10,000\text{ g}$  for 15 min at  $4^{\circ}\text{C}$ . All passages were carried out on ice. All supernatants were quantified for protein content (Coomassie Blue reagent was from Bio-Rad Laboratories).

### Western blot analysis

Lysates (25  $\mu\text{g}$  of protein) were separated by SDS/PAGE and transferred onto PVDF (Immobilon, Millipore), as previously described ([Rapizzi \*et al.\* 2015](#)). Bound antibodies were detected using ECL reagents (Immobilon, Millipore) and analyzed with a Bio-rad ChemiDoc Imaging System for dedicated chemiluminescent image acquisition (Quantity-One Imaging and Analysis Software by Bio-Rad). Primary antibodies against subunit A of SDH (1:5000 dilution; ab14715, Abcam), actin (1:5000 dilution; sc-1615, Santa Cruz Biotechnology) and TOM20 (1:5000 dilution; cat. 612278, BD Biosciences) were used.

Secondary antibodies were obtained from Santa Cruz Biotechnology (anti-mouse sc-2005, anti-goat sc-2020).

### SDH activity

Tissue homogenates (50 µg) were incubated in a phosphate buffer containing sodium azide, 2,6-dichlorophenolindophenol (DCPIP), sodium succinate and phenazine methosulfate. Complex II specific activity was evaluated by photometry, using the Victor<sup>3</sup> 1420 Multilabel Counter (Packard Instruments, Perkin-Elmer), measuring the decrease in absorbance due to oxidation of DCPIP at 600 nm (Rapizzi *et al.* 2015).

### Intracellular ATP

ATP levels were measured using CellTiter-Glo luminescent cell viability assay (Promega). Briefly, muscle homogenates (25 µg/50 µL) were plated in duplicates in a 96-well plate, and an equal volume of CellTiter-Glo reagent was added. Suspensions were shaken at 300 rpm on an orbital shaker for 5 min and then incubated at room temperature for 25 min to stabilize the luminescent signal. Luminescence was measured using the Victor<sup>3</sup> 1420 Multilabel Counter (Packard Instruments, Perkin-Elmer).

### Statistical analysis

Statistical analysis was performed with a one-way ANOVA test (Kruskal–Wallis) followed by Mann–Whitney *post hoc* analysis or with unpaired two-sided Student's *t*-test, respectively, for non-normally and normally distributed parameters, to evaluate differences between groups. Results are expressed as mean ± s.e.m. for normally distributed parameters and median with interquartile range for parameters with non-normal distributions. Correlations were assessed using Spearman's methods. Multilevel mixed-effects linear regressions were used for assessing the acute effect of physical exercise over the running and the recovery time in the experimental groups. The models, nested within the animals, were fitted using the treatment group (RD+PhyEx, HFD+PhyEx or HFD+testosterone+PhyEx), time of exercise (pre-exercise, end exercise and recovery) and their interaction term as the independent variables and the serum parameters as the dependent variables. A *P* value <0.05 was considered as significant in all the analyses. Statistical analysis was performed with Statistical Package for the Social Sciences (SPSS v.25.0; SPSS Inc.).

Experimental procedures were carried out using the facilities of the Molecular Medicine Facility, Department of Biomedical Experimental and Clinical Sciences 'Mario Serio', University of Florence.

## Results

### MetS phenotype induced by HFD

HFD rabbits showed a significant increase in all MetS components. In fact, as shown in Table 1, they have hyperglycemia, glucose intolerance, dyslipidemia, hypertension and increase in visceral fat. More than three MetS components were present in 72.7% of HFD-treated rabbits, whereas it was completely absent in RD rabbits with or without PhyEx (*P*<0.0001). Testosterone supplementation to HFD rabbits significantly decreased the percentage of rabbits satisfying MetS criteria (9.1%, *P*=0.002). This percentage was further reduced to 0% in HFD rabbits performing PhyEx and treated with testosterone (Table 1). PhyEx alone numerically reduced the percentage of MetS rabbits (66.7%), but the difference was not statistically significant from the HFD group (*P*=0.79).

The occurrence of a HFD-associated hypogonadotropic hypogonadism was demonstrated by the significant reduction of both circulating testosterone and LH levels (Table 1). Treatment with testosterone (TTh; 30 mg/kg/week for 12 weeks) did not affect HFD-induced dyslipidemia, while significantly ameliorated MAP, and exerted a remarkable positive effect on fasting glucose levels, glucose tolerance, and visceral adipose tissue accumulation (Table 1). As expected, in testosterone-treated rabbits, LH levels remained suppressed (Table 1). Moreover, no significant differences were observed concerning body weight measurements among the different groups (Table 1). Daily food intake in HFD-fed rabbits was not statistically different, as compared to RD-fed rabbits (127.85 g (108.04–150.48) and 138.7 g (121.7.50–155.61), respectively; *P*=0.154).

### Effects of HFD and TTh on skeletal muscle composition

To characterize the fiber type composition of skeletal muscle samples from each experimental group, we analyzed the mRNA expression of myosin heavy chain (MYH) isoforms, responsible for the contractile properties. We therefore investigated the four fiber types in

**Table 1** Metabolic parameters of experimental groups at week 12.

	Without PhyEx			With PhyEx		
	RD (n = 15)	HFD (n = 15)	HFD + testosterone (n = 15)	RD (n = 10)	HFD (n = 12)	HFD + testosterone (n = 10)
Total body weight (kg)	3.85 (3.66–3.90)	3.80 (3.64–3.96)	3.81 (3.54–3.98)	3.85 (3.60–4.24)	3.52 (3.45–3.79)	3.56 (3.51–3.77)
Blood glucose (g/L)	1.02 (0.99–1.18)	2.03 (1.80–2.28) <sup>c</sup>	1.29 (0.96–1.48) <sup>f</sup>	1.02 (0.88–1.12)	1.84 (1.50–2.22)	1.38 (1.08–1.62) <sup>j</sup>
OGTT (iAUC)	128.60 (109.10–158.00)	211.60 (185.00–224.90) <sup>c</sup>	173.20 (107.82–188.55) <sup>e</sup>	163.15 (114.97–174.02)	232.45 (190.87–266.07) <sup>h</sup>	174.65 (151.05–223.40)
Cholesterol (mg/dL)	49.00 (47.00–50.00)	1314.00 (1179.00–1473.00) <sup>c</sup>	1202.00 (994.00–1316.00) <sup>c</sup>	50.00 (49.00–50.00)	752.52 (443.25–1193.25) <sup>e,i</sup>	652.50 (489.75–900.00) <sup>k</sup>
Triglycerides (mg/dL)	80.00 (57.00–110.00)	156.00 (102.00–201.00) <sup>b</sup>	134.00 (86.00–346.00) <sup>b</sup>	60.00 (36.00–76.50)	150.00 (90.75–245.75) <sup>h</sup>	132.00 (81.00–216.00) <sup>h</sup>
MAP (mmHg)	81.25 (70.00–105.00)	132.00 (120.00–162.50) <sup>c</sup>	106.25 (104.00–120.00) <sup>b,e</sup>	100.00 (80.00–105.62)	125.00 (125.00–175.00)	112.50 (101.87–123.12) <sup>g,l</sup>
VAT (g/kg of total weight)	9.03 (7.07–9.78)	11.25 (10.43–12.07) <sup>c</sup>	1.05 (0.63–1.62) <sup>c,f</sup>	8.88 (7.89–9.96)	9.60 (6.69–10.50) <sup>d</sup>	2.04 (1.15–2.92) <sup>m</sup>
Testosterone (nmoles/L)	5.40 (4.00–7.60)	1.00 (0.69–1.80) <sup>c</sup>	14.70 (9.08–35.20) <sup>b,f</sup>	7.00 (3.90–7.45)	13.75 (2.55–5.55) <sup>f</sup>	39.00 (21.80–52.00) <sup>l,m</sup>
LH (ng/mL)	0.40 (0.18–0.66)	0.15 (0.12–0.26) <sup>a</sup>	0.10 (0.06–0.20) <sup>b</sup>	0.32 (0.17–0.51)	0.65 (0.43–0.77) <sup>g</sup>	0.07 (0.04–0.10) <sup>h,m</sup>
Prevalence of MetS	0	72.7% <sup>c</sup>	9.1% <sup>e</sup>	0	66.7%	0

Results are reported as medians with quartiles in brackets.

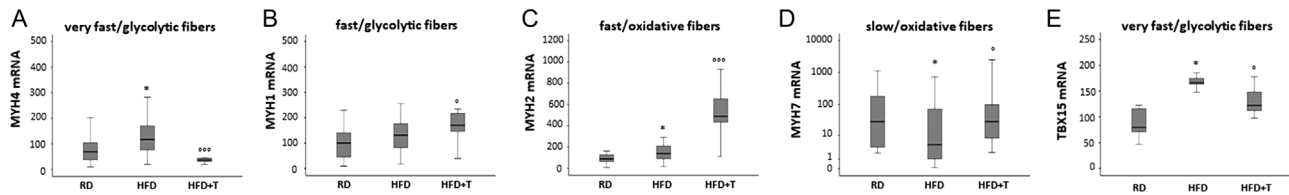
<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs RD; <sup>c</sup>*P* < 0.05, <sup>d</sup>*P* < 0.01 vs HFD; <sup>e</sup>*P* < 0.05, <sup>f</sup>*P* < 0.01 vs RD + PhyEx; <sup>g</sup>*P* < 0.05, <sup>h</sup>*P* < 0.01 vs HFD + testosterone; <sup>i</sup>*P* < 0.05, <sup>j</sup>*P* < 0.001 vs HFD + PhyEx. iAUC, incremental area under the curve of glucose blood level during OGTT; OGTT, oral glucose tolerance test; MAP, mean arterial pressure; VAT, visceral adipose tissue.

mammalian muscle (type I, IIA, IIX, and IIB), according to the expression of MYH isoform: MYH4 for IIB (very fast/glycolytic), MYH1 for IIX (fast/glycolytic), MYH2 for IIA (fast/oxidative) and MYH7 for type I (slow/oxidative). As shown in Fig. 1, mRNA levels of MYH4 and MYH2 (isoforms specific for type IIB and IIA fibers, respectively) were significantly increased in HFD rabbits when compared to RD group (Fig. 1A and C; *P* < 0.05). In contrast, HFD muscle samples showed a significant decrease of the type I marker MYH7 (Fig. 1D; *P* < 0.05 vs RD). TTh significantly counteracted the HFD-induced changes for both MYH4 and MYH7 mRNA expression (Fig. 1A and D; *P* < 0.001 and *P* < 0.05 vs HFD, respectively), while increased MYH1 and further increased MYH2 mRNA (Fig. 1B and C; *P* < 0.05 and *P* < 0.001, respectively). Moreover, the *TBX15* gene, encoding a transcription factor specifically expressed in glycolytic myofibers, resulted increased in HFD samples (Fig. 1E; *P* < 0.05 vs RD) and decreased up to the control level by TTh (Fig. 1E; *P* < 0.05 vs HFD).

Histomorphological analysis by PAS staining of muscle cross-sections confirmed results from mRNA expression. Based on PAS staining intensity, indicative of glycogen content, fibers were classified as light-, medium- or dark-stained, ranging from oxidative to glycolytic. The staining showed a pronounced shift from light PAS-stained (oxidative) to medium/dark PAS-stained (glycolytic) fibers in muscle sections from HFD animals (Fig. 2). In particular, HFD muscles exhibited a significant reduction of light PAS-stained fibers (*P* < 0.001) and a significant increase in medium PAS-stained fibers (*P* < 0.01), as compared to RD muscles (Fig. 2D). TTh significantly increased light-stained fibers (Fig. 2D; *P* < 0.05 vs RD, *P* < 0.001 vs HFD) and decreased medium-stained fibers (Fig. 2D; *P* < 0.05 vs RD, *P* < 0.001 vs HFD). These changes in fiber type composition were confirmed by measuring SDH enzyme activity as marker of oxidative fibers using a specific staining on muscle sections (Fig. 2E, F, G and H). In HFD muscle sections, the percentage of SDH-positive fibers was significantly reduced as compared to RD samples (Fig. 2H; *P* < 0.001) and significantly increased by TTh (Fig. 2H; *P* < 0.01).

We next investigated the effect of HFD and TTh on fiber diameter and markers of muscle atrophy and regeneration. As shown in Fig. 3, HFD reduced fiber diameter (*P* < 0.001 vs RD; Fig. 3A) and increased the mRNA expression of the atrophy-related genes, such as *FBX032/Atrogin 1* (*P* < 0.01 vs RD; Fig. 3B) and *MURF1* (*P* < 0.01 vs RD; Fig. 3C). TTh counteracted the HFD-related reduction of fiber diameter (*P* < 0.001 vs HFD, *P* < 0.05 vs RD; Fig. 3A) and normalized





**Figure 1**

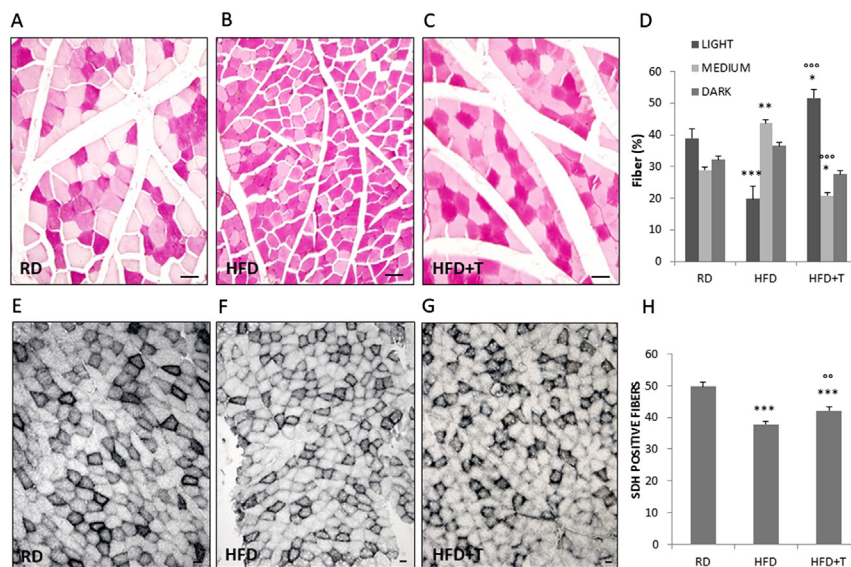
Effect of HFD and TTh on mRNA expression of fiber type markers. (A, B, C and D) mRNA expression of MYH isoforms: MYH4 for type IIB/very fast glycolytic fibers (A), MYH1 for type IIX/fast glycolytic fibers (B), MYH2 for type IIA/fast oxidative fibers (C) and MYH7 for type I/slow oxidative fibers (D). (E) mRNA expression of the fast glycolytic myofiber marker TBX15. The analyses were performed by quantitative RT-PCR in rabbit skeletal muscle from RD, HFD and HFD + testosterone groups. Data were calculated using the  $2^{-\Delta\Delta Ct}$  comparative method, with the 18S ribosomal RNA subunit used as reference gene for normalization, and are reported as mean  $\pm$  s.e.m. ( $n = 15$  for each group). Statistical analysis between groups was performed with Kruskal–Wallis and *post hoc* Mann–Whitney non-parametric tests (\* $P < 0.05$  vs RD; ° $P < 0.05$ , °° $P < 0.001$  vs HFD).

FBX032 mRNA expression up to RD level ( $P < 0.001$  vs HFD; Fig. 3B). Moreover, TTh significantly reduced MURF1 mRNA expression as compared to HFD ( $P < 0.05$ ; Fig. 3C). Interestingly, MYH4 and TBX15 expression (markers of glycolytic fibers) was positively associated with expression of FBX032/Atrogin 1 mRNA ( $r = 0.408$ ,  $P = 0.015$ ,  $n = 35$  and  $r = 0.458$ ,  $P = 0.049$ ,  $n = 19$ , respectively).

On the other side, the myogenesis-related gene myogenin (MYOG) was significantly decreased in HFD muscles (Fig. 3D;  $P < 0.001$  vs RD) and restored by TTh (Fig. 3D,  $P < 0.01$  vs HFD). TTh also upregulated the mRNA expression of two other myogenesis markers, such as MYF5 (Fig. 3E,  $P < 0.001$  vs RD and HFD) and PAX7 (Fig. 3F,  $P < 0.01$  vs RD and HFD) and the muscle differentiation markers MYOD (Fig. 3K,  $P < 0.01$  vs RD

and HFD) and ITGA7/ $\alpha 7$  integrin (Fig. 3L,  $P < 0.001$  vs RD and HFD). The myogenic effect of TTh was confirmed by immunohistochemistry of PAX7 expression in skeletal muscle sections from each rabbit group. As shown in Fig. 3 (panels G, H, I and J), we found a significant increase of PAX7-positive cells in muscle sections from testosterone-treated rabbits, as compared to both RD and HFD groups ( $P < 0.001$ ). Moreover, using an antibody against MYH3, which is expressed by newly generated myofibers, we detected MYH3-positive fibers exclusively in muscle sections from testosterone-treated HFD rabbits (Fig. 3M).

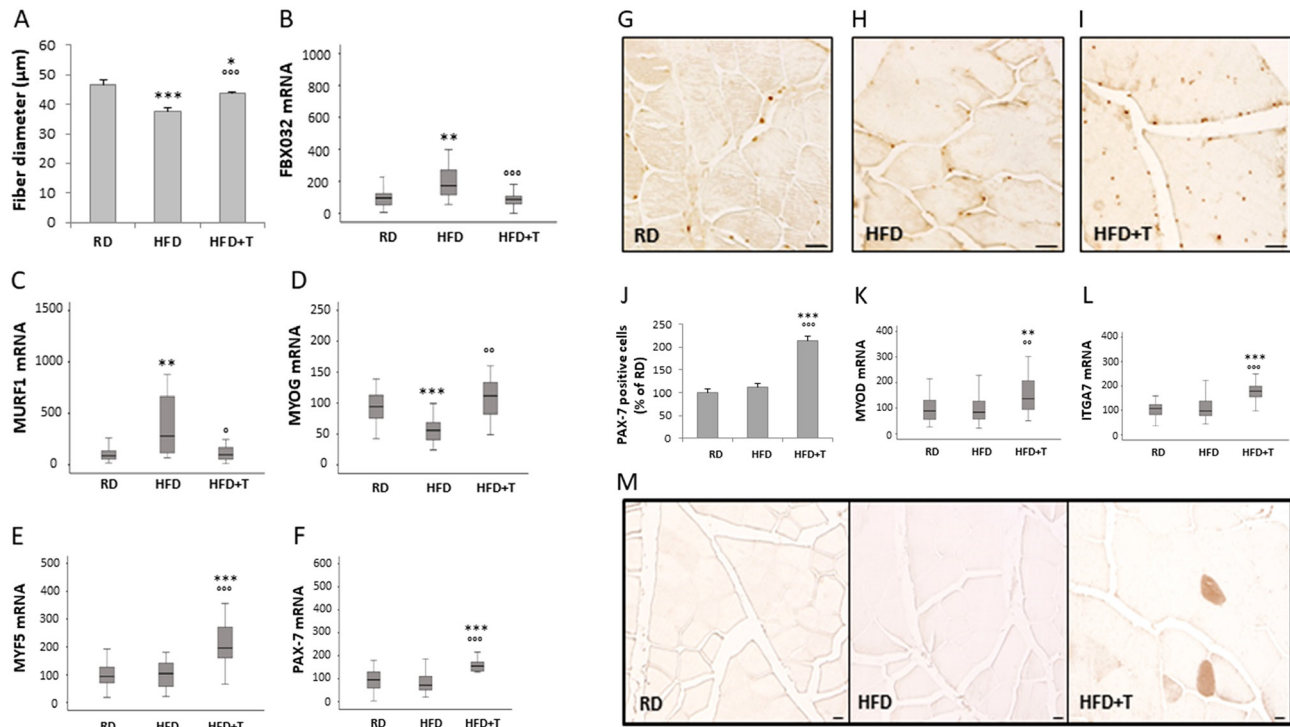
Accordingly, a positive association was found between circulating testosterone and skeletal muscle expression of several genes related to muscle regeneration (MYOG, MYF5, PAX7, ITGA7), mitochondrial and oxidative metabolism (SLC25A12, NDUFB5, UCP2), while the expression of the



**Figure 2**

Effect of HFD and TTh on muscle fiber composition. (A, B and C) Representative images of PAS-stained vastus medialis cross-sections from RD (A), HFD (B) and HFD + testosterone (C) rabbits showing a shift toward glycolytic more intense stained fibers in HFD samples compared to RD, that was reverted by TTh; (D) quantification of light, medium and dark stained fibers calculated by counting ten fields from muscle sections of at least three animals for each experimental group. (E, F and G) Representative images of SDH enzymatic assay in vastus medialis cryo-sections from RD (E), HFD (F) and HFD + testosterone (G) rabbits showing a reduction of SDH activity in HFD sections, significantly counteracted by TTh. (H) Quantification of SDH-positive fibers calculated by counting ten fields of at least three animals for each experimental group. Statistical analysis between groups was performed with unpaired two-sided Student's *t*-test (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs RD; °° $P < 0.01$ , °°° $P < 0.001$  vs HFD). Scale bar 50  $\mu$ m. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0532>.





**Figure 3**

Effect of HFD and TtH on fiber size and muscle regeneration/differentiation. (A) Fiber diameter, as measured in muscle cross-sections of specimens from RD, HFD and HFD + testosterone rabbits, was reduced by HFD, while TtH counteracted this effect. Data were calculated by ImageJ 1.51f software in ten fields from at least three animals for each experimental group and expressed as mean  $\pm$  s.e.m. Statistical analysis between groups was performed with unpaired two-sided Student's *t*-test ( $*P < 0.05$  and  $***P < 0.001$  vs RD,  $^{\circ\circ\circ}P < 0.001$  vs HFD). (B, C, D, E, F and G) TtH normalized the HFD-altered mRNA expression of atrophy-related genes, such as FBX032/Atrogin-1 (B) and MURF-1 (C), and the myogenesis-related gene MYOG (D). Also the muscle regeneration markers MYF5 (E) and PAX7 (F), the muscle differentiation markers MYOD (K) and ITGA7/ $\alpha 7$  integrin (L) were significantly increased by TtH. Data were calculated using the  $2^{-\Delta\Delta Ct}$  comparative method, with the 18S ribosomal RNA subunit used as reference gene for normalization and are reported as mean  $\pm$  s.e.m. ( $n = 15$  for each group). Statistical analysis was performed with Kruskal-Wallis and *post hoc* Mann-Whitney non-parametric tests ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  vs RD;  $^{\circ\circ}P < 0.01$ ,  $^{\circ\circ\circ}P < 0.001$  vs HFD). (G, H, I and J) Immunohistochemical analysis of PAX-7 in vastus medialis muscle cross-sections from RD (G), HFD (H) and HFD + testosterone (I) rabbits; (J) quantification of PAX-7-positive cells counted in ten fields from three animals for each group and expressed as percentage of RD (mean  $\pm$  s.e.m.), showing a significant increase of PAX-7 expression in sections from testosterone-treated HFD rabbits (scale bar 20  $\mu$ m). Statistical analysis between groups was performed with unpaired two-sided Student's *t*-test ( $***P < 0.001$  vs RD;  $^{\circ\circ\circ}P < 0.001$  vs HFD). (M) Representative images of the muscle regeneration marker MYH3 in vastus medialis cross-sections, as evaluated by immunohistochemical analysis: MYH3-positive fibers are detected exclusively in HFD + testosterone sample (scale bar 20  $\mu$ m). A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0532>.

type IIB marker MYH4 and atrophy marker FBX032 was negatively associated with testosterone levels (Table 2).

As reported in Fig. 4, TtH effect on oxidative function was confirmed by the significant induction of the expression of some mitochondrial respiration-related genes, such as NADH dehydrogenase ubiquinone 1 beta subcomplex 3 and 5 (NDUF3 and NDUF5;  $P < 0.001$  and  $P < 0.01$  vs both RD and HFD, respectively; Fig. 4A and B), NADH-ubiquinone oxidoreductase subunit 1 (NDUF1;  $P < 0.05$  vs both RD and HFD; Fig. 4C), succinate dehydrogenase (SDHB;  $P < 0.01$  vs both RD and HFD; Fig. 4D) and citrate synthase (CS;  $P < 0.01$  vs RD,  $P < 0.05$  vs HFD; Fig. 4E). Accordingly, transmission electron microscopy studies evidenced mitochondrial dysfunctions in HFD muscle samples, with pronounced

reduction of the internal cristae surface area, as compared to RD (Fig. 4F, G and I;  $P < 0.01$  vs RD). TtH counteracted the HFD-induced modifications of the mitochondrial cristae (Fig. 4H and I;  $P < 0.05$  vs HFD).

To further characterize skeletal muscle metabolism, we analyzed the mRNA expression of genes related to insulin sensitivity, lipid and energy metabolism (Fig. 5). We found that TtH induced a significant increase in mRNA expression of insulin receptor substrate-1 (IRS-1;  $P < 0.001$  vs RD and HFD; Fig. 5A), lipoprotein lipase (LPL;  $P < 0.01$  vs RD,  $P < 0.05$  vs HFD; Fig. 5B), peroxisome proliferator-activated receptor- $\alpha$  (PPAR $\alpha$ ;  $P < 0.001$  vs RD and HFD; Fig. 5C), uncoupled protein-2 (UCP2;  $P < 0.001$  vs RD and HFD; Fig. 5D) and UCP3 ( $P < 0.01$  vs RD,  $P < 0.05$  vs HFD; Fig. 5E). Interestingly, the brown adipose

**Table 2** Association between gene expression in skeletal muscle samples and circulating testosterone.

Gene	<i>r</i>	<i>P</i>	<i>n</i>
<i>MYF5</i> (myogenic factor 5)	0.537	<b>0.000</b>	45
<i>PAX7</i> (paired box 7)	0.390	<b>0.008</b>	45
<i>MYOG</i> (myogenin)	0.472	<b>0.003</b>	38
<i>ITGA7</i> (skeletal muscle transcripts of the human integrin alpha7)	0.539	<b>0.000</b>	45
<i>SLC25A12</i> (solute carrier family 25 aspartate/glutamate carrier, member 12)	0.386	<b>0.009</b>	45
<i>NDUFB5</i> (NADH dehydrogenase ubiquinone 1 beta subcomplex 5)	0.444	<b>0.002</b>	45
<i>MYH4</i> (myosin, heavy chain 4)	-0.424	<b>0.004</b>	45
<i>FBX032</i> (muscle atrophy F-box)	-0.368	<b>0.013</b>	45
<i>UCP2</i> (uncoupling protein 2)	0.516	<b>0.000</b>	45

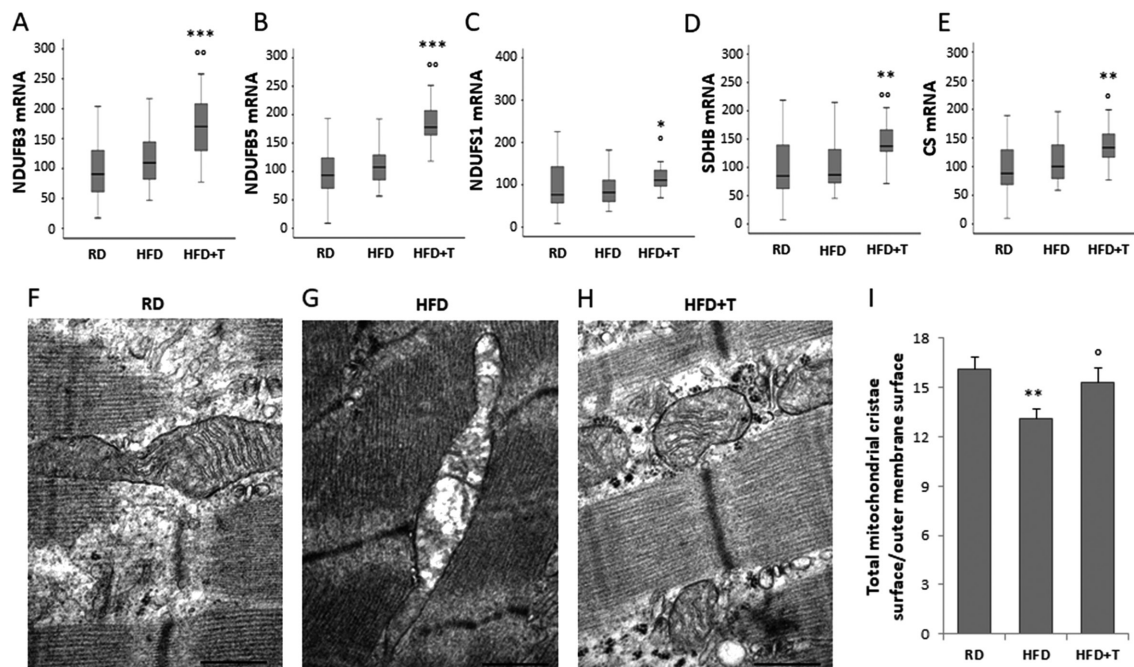
Results derived from univariate correlations and are presented as Spearman coefficients. Bold indicates statistical significance.

tissue-specific UCP1 mRNA was significantly reduced by HFD ( $P < 0.05$  vs RD; Fig. 5F) and normalized by TTh ( $P < 0.01$  vs HFD; Fig. 5F). Moreover, Oil Red O staining showed that skeletal muscle cryosections from HFD rabbits presented a lipid intramyocellular accumulation (Fig. 5H), which was not present in muscle samples from RD, where a light lipid positivity was localized to intermuscular spaces (Fig. 5G). These results were paralleled by a significant increase of triglyceride content in HFD muscle extracts

as compared to RD group ( $P < 0.05$ ; Fig. 5J). Interestingly, TTh normalized both lipid content localization (Fig. 5I) and triglyceride content ( $P < 0.05$  vs HFD; Fig. 5J).

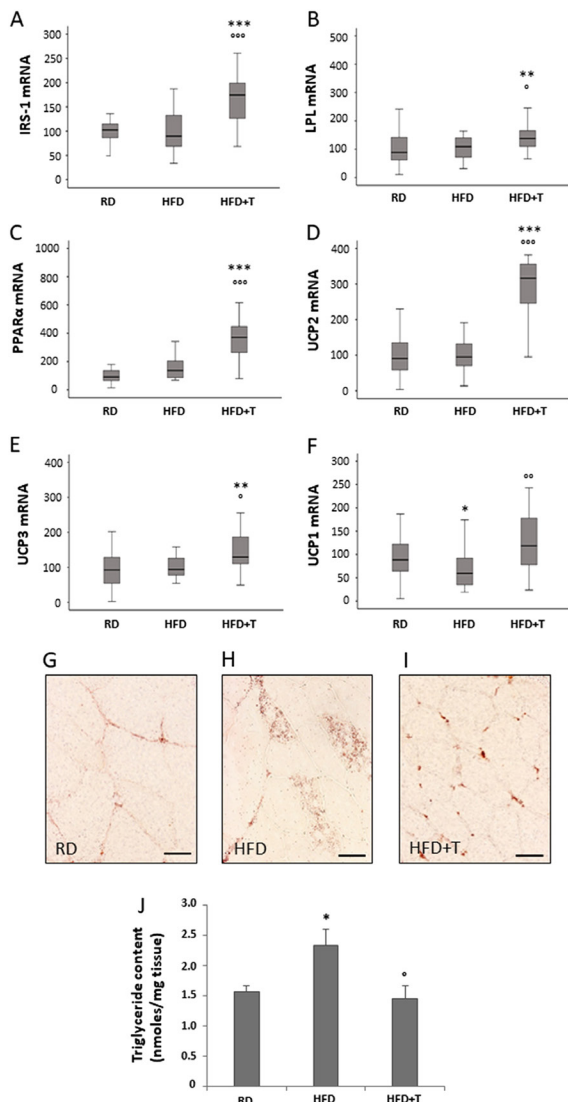
### Effect of physical exercise on skeletal muscle composition and metabolism

Since we observed alterations in skeletal muscle induced by HFD and ameliorated by TTh, we next evaluated the



**Figure 4**

Effect of HFD and TTh on mitochondrial respiration-related gene expression and mitochondrial cristae aspect. (A, B, C, D and E) Quantitative RT-PCR analysis shows a positive effect of TTh on mRNA expression of the mitochondrial respiration-related genes NDUFB3 (A), NDUFB5 (B), NDUFS1 (C), SDHB (D) and CS (E). Data were calculated using the  $2^{-\Delta\Delta Ct}$  comparative method, with the 18S ribosomal RNA subunit used as reference gene for normalization, and are reported as mean  $\pm$  s.e.m. ( $n = 15$  for each group). Statistical analysis was performed with Kruskal-Wallis and *post hoc* Mann-Whitney non-parametric tests (\*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs RD,  $^{\circ}P < 0.05$ ,  $^{\circ\circ}P < 0.01$ ,  $^{\circ\circ\circ}P < 0.001$  vs. HFD). (F, G, H and I) Mitochondrial ultrastructure, as visualized by transmission electron microscopy in skeletal muscle specimens, was affected by HFD (G) and normalized by TTh (H). Bar graph (I) represents the ratio between the cristae membrane surface area and the outer membrane surface area as measured using iTEM image analysis software (SIS) in ten fields of muscle sections from at least three animals for each experimental group. Data are reported as mean  $\pm$  s.e.m. Statistical analysis was performed with unpaired two-sided Student's *t*-test (\*\* $P < 0.01$  vs RD;  $^{\circ}P < 0.05$  vs HFD). Scale bar 500 nm.



**Figure 5**

TTh effect on genes related to insulin sensitivity, lipid and energy metabolism and on lipid accumulation in muscle samples. (A, B, C, D, E and F) TTh induced mRNA expression of IRS-1, gene related to insulin-dependent signaling (A), as well as LPL (B) and PPARα (C), genes implicated in the lipid metabolism. TTh also increased the expression of genes involved in energy dissipation as UCP2 (D) and UCP3 (E) and normalized the HFD-altered expression of the brown adipose tissue-specific UCP1 (F). Data were calculated using the  $2^{-\Delta\Delta Ct}$  comparative method, with the 18S ribosomal RNA subunit used as reference gene for normalization, and are reported as mean  $\pm$  s.e.m. ( $n = 15$  for each group). Statistical analysis between groups was performed with Kruskal–Wallis and *post hoc* Mann–Whitney non-parametric tests ( $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  vs RD;  $^{\circ}P < 0.05$ ,  $^{\circ\circ}P < 0.01$ ,  $^{\circ\circ\circ}P < 0.001$  vs HFD). (G, H and I) Oil Red O staining shows intramyocellular lipid accumulation in HFD muscle cryosections (H) and intermuscular lipid localization in RD (G) and HFD + testosterone (I) muscle sections (scale bar 50  $\mu$ m). (H) TTh normalized the triglycerides content increased by HFD, as evaluated by Triglyceride Quantification Kit on muscle lysates from six animals for each experimental group; data are reported as mean  $\pm$  s.e.m. and statistical analysis was performed with unpaired two-sided Student's *t*-test ( $*P < 0.05$  vs RD,  $^{\circ}P < 0.05$  vs HFD). A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0532>.

physical endurance and the skeletal muscle functional response in terms of fiber composition and metabolism in all the experimental groups. We trained a subgroup of RD, HFD and HFD+testosterone rabbits with a progressive treadmill running protocol for 12 weeks. In HFD rabbits, physical activity significantly improved MetS parameters, such as hypercholesterolemia, percentage of visceral fat and testosterone and LH levels when compared to the untrained rabbits (Table 1). Conversely, no effect was observed in glucose metabolism, triglycerides and mean arterial pressure (Table 1). As expected, in testosterone-treated rabbits, physical activity did not change LH levels that remained suppressed (Table 1). Although the HFD+testosterone+PhyEx group showed numerical improvements in some MetS components (such as blood glucose, MAP and VAT), the differences were not statistically significant, as compared to the HFD+testosterone group (Table 1).

Maximum running time and distance on the treadmill were utilized to assess the endurance capacity of rabbits, exercised on a 20% slope until unable to run for 10 s. As showed in Table 3, HFD rabbits were able to run for a lower distance and for a shorter time respect to RD rabbits, although the lactate production was similar in the two groups. When lactate production was normalized for running distance, it resulted significantly increased by HFD (Table 3). TTh significantly improved physical resistance to exercise by increasing both maximal running time and distance and normalizing lactate production over running distance (Table 3). Table 3 also shows glycemia levels before and after exercise. In RD+PhyEx at the end of exercise glycemia tends to increase, whereas in HFD+PhyEx rabbits such an increase was not observed, although the pre-exercise levels were higher than in control. TTh partially reduced basal glycemia and allowed a sustained increase in glycemia upon PhyEx.

The effect of endurance training on fiber muscle composition was evaluated by PAS staining and SDH activity assay (Fig. 6). In HFD+PhyEx muscles, PAS staining showed a shift to glycolytic fibers with an increase in both medium and dark staining and a parallel reduction of light stained fibers (Fig. 6A and B) when compared to RD+PhyEx ( $P < 0.001$  and  $P < 0.05$  vs RD+PhyEx, respectively, see bar graph in Fig. 6D). TTh was able to restore all these alterations up to RD+PhyEx levels (Fig. 6C and D). These data were confirmed by measuring SDH enzyme activity with a specific staining on muscle sections (Fig. 6E, F, G and H) and with an enzymatic assay from total muscle extracts (Fig. 6I). In HFD+PhyEx samples,

**Table 3** Effects of acute exercise in trained RD, HFD and HFD + testosterone rabbits.

	Pre-exercise	End	P end vs pre-exercise within group	Recovery	P recovery vs pre-exercise within group
Running distance (m)					
RD	---	200.00 (159.87; 308.13)	---	---	---
HFD	---	105.00 (76.88; 154.78) <sup>b</sup>	---	---	---
HFD + testosterone	---	225.00 (164.87; 338.50)	---	---	---
Running time (s)					
RD	---	600.00 (479.62; 924.38)	---	---	---
HFD	---	315.00 (230.65; 464.35) <sup>b</sup>	---	---	---
HFD + testosterone	---	675.00 (494.60; 1015.51)	---	---	---
Lactate (nmol/L)					
RD	3.40 (2.38; 6.75)	14.55 (10.37; 19.87)	<0.0001	---	---
HFD	3.10 (1.86; 7.83)	18.30 (12.42; 20.14)	<0.0001	---	---
HFD + testosterone	7.20 (5.02; 10.48)	18.35 (14.07; 21.73)	<0.0001	---	---
Lactate/running distance (nmol/L for each 100 m)					
RD	---	6.16 (4.15; 11.95)	---	---	---
HFD	---	16.18 (11.65; 22.73) <sup>b</sup>	---	---	---
HFD + testosterone	---	7.36 (5.29; 12.11)	---	---	---
Blood glucose (g/L)					
RD	0.95 (0.76; 1.11)	1.16 (0.92; 1.48)	0.086	1.02 (0.91; 1.15)	0.553
HFD	1.60 (1.33; 2.26) <sup>c</sup>	1.94 (1.65; 2.15) <sup>b</sup>	0.449	1.84 (1.52; 2.29) <sup>c</sup>	0.537
HFD + testosterone	1.26 (1.08; 1.68)	1.73 (1.21; 2.69) <sup>b</sup>	<0.0001	1.38 (1.06; 1.60) <sup>a</sup>	0.223
Triglycerides (mg/dL)					
RD	59.50 (51.50; 81.90)	66.00 (52.77; 86.63)	0.727	60.00 (43.50; 72.50)	0.311
HFD	139.50 (71.04; 342.96) <sup>a</sup>	155.50 (67.52; 355.65) <sup>a</sup>	0.559	162.00 (72.29; 350.38) <sup>b</sup>	0.581
HFD + testosterone	111.50 (70.22; 217.18)	120.00 (62.23; 215.77)	0.584	132.00 (87.89; 212.51)	0.449

Measurements were performed before starting (pre-exercise), at the end of the exercise (end) and after 90' from the exercise (recovery). Results derive from multilevel mixed-effect linear regressions (nested within the animals) using the serum parameters as dependent variables and treatment, time of exercise and their interaction term as independent variables. The interaction time × treatment is reported using RD as referent. Data are reported as median with 95% confidence intervals (in brackets). RD, *n* = 10; HFD, *n* = 12 and HFD + testosterone, *n* = 10.

<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>c</sup>*P* < 0.0001 vs RD.

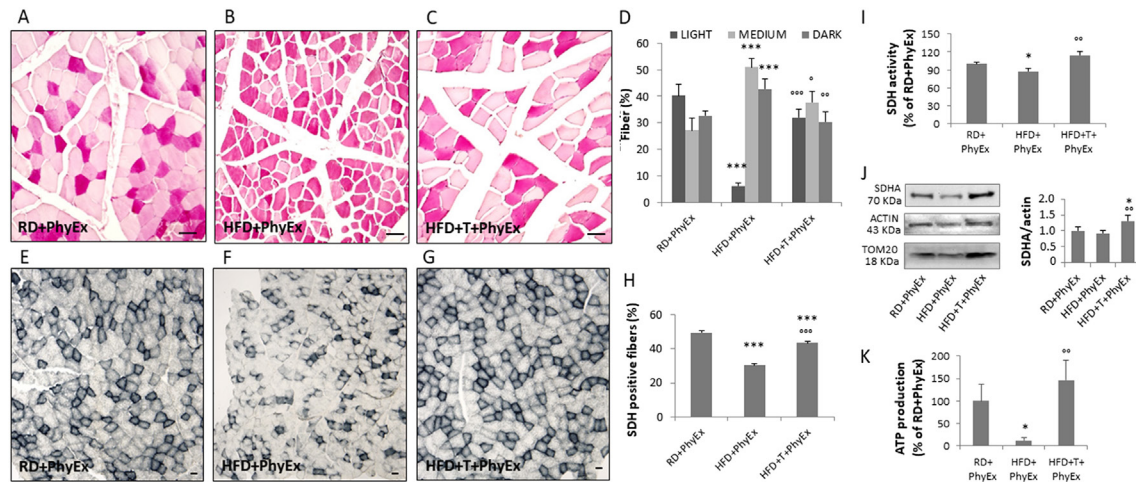
the activity of SDH was reduced and restored by TTh (Fig. 6E, F, G, H and I). Moreover, also the protein expression of the subunit A of SDH, evaluated by Western blotting of vastus medialis lysates, was significantly increased by TTh (Fig. 6J, *P* < 0.01 vs either RD+PhyEx or HFD+PhyEx). Similar results were obtained analyzing the protein expression of TOM20, a marker of mitochondrial mass (Fig. 6J). To further demonstrate the positive effect of TTh on mitochondrial respiration, we measured ATP levels in muscle extracts from each experimental trained group. Figure 6K shows a significant reduction of ATP levels in HFD+PhyEx samples, as compared to RD+PhyEx (*P* < 0.01), that was completely reverted by TTh (*P* < 0.01 vs HFD+PhyEx).

Transmission electron microscopy analysis of mitochondria further demonstrated the negative effects of HFD on cristae that were maintained also in exercise trained rabbits (Fig. 7A and B; *P* < 0.001 vs RD+PhyEx); once again TTh was able to ameliorate mitochondrial cristae aspect (Fig. 7C; *P* < 0.05 vs HFD+PhyEx).

### Effect of testosterone supplementation on skeletal muscle composition and physical endurance in RD group

To evaluate whether testosterone effects could be detected also in RD animals, independently of the HFD-induced features (including hypogonadism), we analyzed an additional group of eugonadal rabbits fed a RD and treated with testosterone (30 mg/kg) for 12 weeks. As evaluated by both mRNA expression analysis of fiber type markers (Table 4) and PAS staining of muscle cross-sections (Fig. 8A), we found that testosterone supplementation to RD did not change skeletal muscle fiber types proportion when compared to RD untreated group. No differences were also observed in the expression of mitochondrial oxidative function-related genes (Table 4). In contrast, fiber diameter (Fig. 8B) and mRNA expression of markers of both myogenesis and muscle differentiation, such as PAX7 (Fig. 8D) and ITGA7 (Fig. 8E), respectively, were significantly increased by testosterone supplementation,



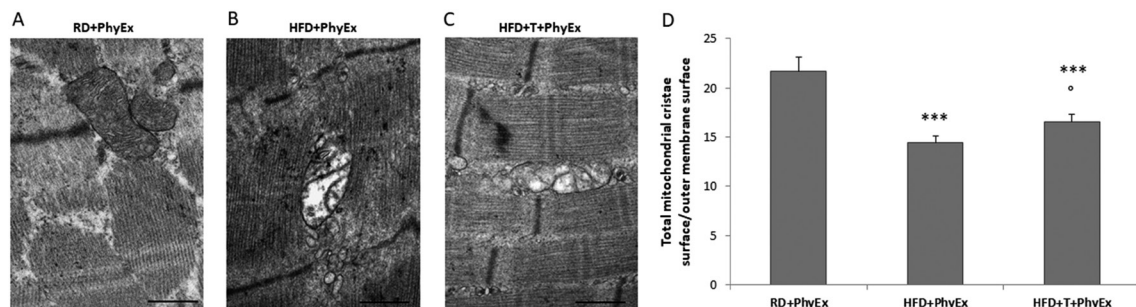


**Figure 6**

Effect of PhyEx and TTH on muscle fiber composition in each experimental group. (A, B and C) Representative images of PAS-stained vastus medialis cross-sections from RD (A), HFD (B) and HFD + testosterone (C) treadmill trained rabbits showing a reduction of light stained fibers and a further shift towards medium/dark stained (glycolytic) fibers induced by HFD and normalized by TTH. (D) Quantification of light, medium and dark stained fibers calculated by counting at least ten fields from muscle sections of at least three different animals for each experimental group. (E, F, G, H and I) SDH enzymatic assay in either vastus medialis cryo-sections or in total muscle extracts shows a reduction of SDH activity in HFD samples, significantly counteracted by TTH. (E, F and G) Representative images of SDH staining in muscle sections. (H) The bar graphs show the quantification of SDH-positive fibers expressed as percentage of total fibers counted in ten fields from at least three different animals for each group. (I) SDH activity in muscle extracts from three animals for each group expressed as percentage of RD + PhyEx, taken as 100%. (J) Representative immunoblots of SDHA and TOM20 in vastus medialis protein lysates from each experimental group and quantification of SDHA band intensity normalized over actin signal; data are expressed as mean  $\pm$  s.e.m. and derive from three different animals for each group. (K) Analysis of ATP levels in muscle lysates from RD, HFD and HFD + testosterone rabbits after PhyEx protocol expressed as percentage over RD + PhyEx, taken as 100%. Data are from at least six animals for each group. Statistical analysis between groups was performed with unpaired two-sided Student's *t*-test (\* $P < 0.05$ , \*\*\* $P < 0.001$  vs RD;  $^{\circ}P < 0.05$ ,  $^{\circ\circ}P < 0.01$ ,  $^{\circ\circ\circ}P < 0.001$  vs HFD). Scale bar 50  $\mu$ m. A full colour version of this figure is available at <https://doi.org/10.1530/JOE-19-0532>.

as compared to RD group ( $P < 0.001$  vs RD for fiber diameter;  $P < 0.01$  vs RD for PAX7 and ITGA7 mRNA). Accordingly, circulating testosterone levels were positively associated with fiber diameter (Fig. 8C) and ITGA7 mRNA expression (Fig. 8F). Genes related to glucose (PPAR $\alpha$  and IRS-1) and energy (UCP1, UCP2 and UCP3) metabolism were not affected by testosterone, except for the triglyceride hydrolysis-related gene LPL, that resulted significantly

increased ( $P < 0.001$  vs RD; Table 4). Moreover, physical endurance evaluation of the subset of RD+testosterone rabbits trained with the treadmill protocol was similar to that of RD group in terms of running distance, running time, lactate production pre- and post-exercise, and lactate/running distance ratio, as reported in Table 5. By analyzing PAS-stained muscle cross-sections, SDH-positive fibers and ATP production in skeletal muscle samples from



**Figure 7**

Effect of PhyEx and TTH on mitochondrial ultrastructure. (A, B and C) Ultrastructural analysis of skeletal muscle specimens showed mitochondrial alterations in HFD samples which were significantly reduced in the samples from TTH-treated animals. (D) Bar graph indicates the ratio between the cristae membrane surface area and the outer membrane surface area as measured using iTEM image analysis software (SIS) in ten fields of muscle sections from at least three animals for each group. Data are reported as mean  $\pm$  s.e.m. Statistical analysis was performed unpaired two-sided Student's *t*-test (\*\*\* $P < 0.001$  vs RD;  $^{\circ}P < 0.05$  vs HFD). Scale bar 500 nm.

**Table 4** Effects of *in vivo* testosterone treatment on mRNA expression of target genes in rabbit skeletal muscle.

Gene	RD	RD + testosterone	P
Fiber types:			
<i>MYH1</i>	100.20 (45.30–143.71)	90.93 (85.42–98.88)	0.822
<i>MYH2</i>	88.67 (60.42–123.63)	132.43 (93.72–166.67)	0.112
<i>MYH4</i>	60.31 (36.87–164.96)	102.96 (70.37–207.14)	0.379
<i>MYH7</i>	32.64 (3.65–186.79)	199.75 (50.30–353.00)	0.223
<i>TBX15</i>	100.43 (75.77–114.24)	93.94 (87.23–147.09)	0.763
Mitochondrial oxidative function:			
<i>CS</i>	88.11 (68.18–129.37)	95.95 (85.99–134.47)	0.510
<i>NDUFB3</i>	93.12 (60.71–130.26)	105.57 (79.02–119.51)	0.749
<i>NDUFB5</i>	95.81 (69.86–122.31)	117.18 (79.64–131.27)	0.560
<i>NDUFS1</i>	76.39 (56.79–143.40)	100.92 (85.39–104.88)	0.533
<i>SDHB</i>	84.91 (59.00–140.35)	105.89 (96.36–114.82)	0.417
<i>SLC25A12</i>	93.68 (76.08–123.21)	107.37 (99.73–114.86)	0.395
Atrophy, myogenesis and muscle differentiation:			
<i>FBXO32</i>	96.03 (51.46–124.81)	116.80 (99.19–123.92)	0.383
<i>MYOG</i>	92.87 (73.56–121.41)	136.88 (94.56–168.03)	0.126
<i>MYF5</i>	92.55 (70.76–129.42)	95.26 (81.81–130.72)	0.704
<b><i>PAX7</i></b>	<b>95.39 (58.55–129.28)</b>	<b>193.65 (138.14–222.75)</b>	<b>0.009</b>
<b><i>ITGA7</i></b>	<b>103.01 (85.64–119.82)</b>	<b>189.02 (137.64–198.60)</b>	<b>0.002</b>
<i>MYOD</i>	88.42 (55.59–131.58)	98.16 (79.95–126.81)	0.778
Glucose, lipid and energy metabolism:			
<i>PPARA</i>	91.40 (64.15–137.61)	122.31 (84.15–147.87)	0.384
<i>IRS1</i>	102.30 (80.32–120.49)	152.17 (100.43–163.82)	0.124
<b><i>LPL</i></b>	<b>88.99 (60.41–142.45)</b>	<b>220.93 (211.07–328.42)</b>	<b>&lt;0.001</b>
<i>UCP1</i>	92.13 (64.90–119.98)	102.72 (71.49–111.62)	0.836
<i>UCP2</i>	90.82 (55.16–135.27)	91.72 (48.56–144.81)	0.953
<i>UCP3</i>	92.20 (53.01–129.61)	86.68 (47.64–131.79)	0.955

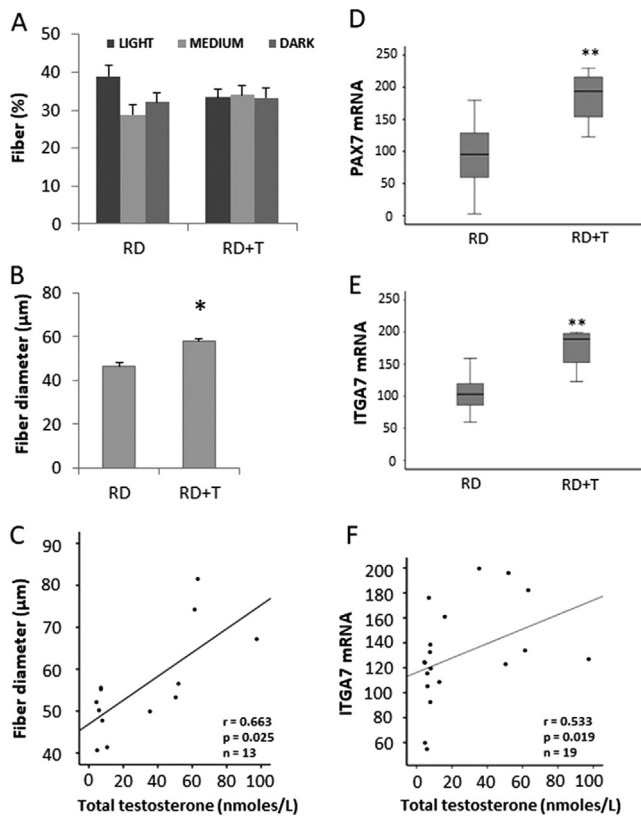
The target genes mRNA expression was evaluated using semi-quantitative RT-PCR in skeletal muscle samples from RD ( $n = 15$ ) and RD + testosterone ( $n = 4$ ) rabbits. Data were calculated according to the comparative  $C_t$  method, using 18S rRNA subunit as the reference gene for normalization. Results are expressed in percentage over RD and are reported as median and quartiles. Bold indicates genes with significantly different expression ( $P < 0.05$ ) comparing RD vs RD + testosterone in the *post hoc* Mann–Whitney analysis.

RD+testosterone+PhyEx rabbits, we confirmed that the global fiber type composition and metabolism of skeletal muscle from RD animals was not significantly affected by testosterone supplementation (data not shown). As shown in Table 6, testosterone supplementation did not change any of the physical and metabolic parameters in RD animals either with or without PhyEx, when compared to their untreated counterpart, apart from the obvious changes in hormonal parameters (Table 6).

## Discussion

Change in lifestyle, which includes the increase of physical activity, is an important and effective strategy to combat MetS-associated hypogonadism (Morelli *et al.* 2019). However, the ability of MetS subjects to perform physical activity might be suboptimal or even compromised, due to a detrimental effect of the syndrome on overall health and, in particular, on body composition. We here demonstrated that TTh in experimental MetS increases

the capacity to perform physical exercise by improving skeletal muscle oxidative activity and ability to sustain endurance exercise. Indeed, physical activity is nowadays regarded as a major intervention for preventing or halting metabolic disturbances, such as those characterizing MetS (Bird & Hawley 2017, Weweg *et al.* 2018). Skeletal muscle represents one of the most important tissues in maintaining normal whole-body metabolism and energy homeostasis because of its high contribution to the whole body mass and to its metabolic capacity (Zurlo *et al.* 1990). In an experimental (HFD-induced) MetS model associated with hypogonadism, we recently described a reduced ability of rabbits to perform physical exercise (Morelli *et al.* 2019). In the present study, we demonstrate that the reduced response to endurance training observed in MetS animals was associated with peculiar HFD-induced modifications in the skeletal muscle fiber composition and metabolism. More relevant, we herein report that when supplemented with testosterone, MetS rabbits exhibited skeletal muscle morphological and functional features similar to those observed in RD animals, including a normalized response



**Figure 8** Effect of TTh on skeletal muscle composition and mRNA expression of regeneration/differentiation markers in RD group. (A) Quantification of light, medium and dark PAS-stained fibers calculated by counting ten fields from muscle sections of at least three animals for each experimental group. (B) Fiber diameter, as measured in muscle cross-sections of specimens from RD + testosterone group compared to RD. Data were calculated by Image J 1.51f software in ten fields from at least three animals for each experimental group and expressed as mean  $\pm$  s.e.m. Statistical analysis between groups was performed with unpaired two-sided Student's *t*-test (\**P* < 0.001). (C) Relationship between fiber diameter and plasma levels of testosterone in the experimental groups, as derived from univariate Spearman's regression analysis. (D and E) TTh in RD rabbits significantly increased mRNA expression of the myogenesis-related gene PAX7 (D), and the muscle differentiation marker ITGA7/ $\alpha$ 7 integrin (E). Data were calculated using the  $2^{-\Delta\Delta Ct}$  comparative method, with the 18S ribosomal RNA subunit used as reference gene for normalization, and are reported as mean  $\pm$  s.e.m. (RD, *n* = 15; RD + testosterone, *n* = 4). Statistical analysis was performed with Kruskal-Wallis and *post hoc* Mann-Whitney non-parametric tests. (\**P* < 0.001). (F) Relationship between ITGA7 mRNA and plasma levels of testosterone in the experimental groups, as derived from univariate Spearman's regression analysis.

to physical activity. On the other hand, testosterone supplementation on otherwise eugonadal rabbits (RD) did not result in significant changes in endurance ability, although increasing muscle fibers diameter. Given the well-known tight link existing between MetS and the development of hypogonadism in male population, our results add novel insights about the importance

of considering TTh as a favorable intervention for the efficacy of physical activity programs in MetS subjects, but not in eugonadal subjects (as it is in doping).

Skeletal muscle is one of the most highly plastic tissues, being capable of extensive remodeling in response to use, disuse and pathological perturbation. In particular, transformations of muscle fiber type may occur to facilitate functional adaptations for a muscle's specific needs, such as those requested during physical activity (Schiaffino & Reggiani 2011). The functional adaptability of skeletal muscle depends upon the type of exercise: resistance exercise increases mass and strength, while endurance exercise can stimulate mitochondrial biogenesis and function, promoting aerobic metabolism. In this regard, endurance exercise has been found to be more effective in preventing cardiovascular and metabolic diseases (Shen *et al.* 2018).

In this study, we analyzed the vastus medialis muscle, a portion of the quadriceps femoris muscle group involved in knee extension, characterized by a mixed composition of type I/type II fibers and therefore useful for studying whether particular conditions could induce changes toward another fiber type. Histological evaluation of HFD muscle samples showed a reduction of slow/type I fibers, which are characterized by the use of oxidative metabolism and the ability to perform long-lasting activities. On the contrary, we demonstrated an increased proportion of fast/glycolytic fibers, which rely on glycolytic metabolism and may not provide prolonged contractions. In fact, the PAS staining showed a reduction of light stained fibers and an increase of the medium/dark stained ones, more rich of glycogen, the substrate of anaerobic contractions. Moreover, HFD induced the expression of the type IIB glycolytic fiber markers MYH4 (Schiaffino & Reggiani 2011) and of the mesodermal transcription factor testosterone-box 15 (TBX15), a highly and specifically expressed transcription factor in glycolytic fast-twitch myofibers (Lee *et al.* 2015). Interestingly, TBX15 ablation determines a shift to oxidative myofibers (Lee *et al.* 2015). The concomitant reduced expression of the type I marker MYH7 in vastus medialis from HFD rabbits further demonstrated a morphofunctional shift in terms of both fiber composition and contractile properties. Interestingly, the aforementioned fiber type modifications were accompanied by muscle atrophy and by an impairment of myogenic capacity, as demonstrated by the reduction of fiber diameter, by the increase of the atrophy-related gene FBX032 and by the reduction of myogenin gene expression in HFD muscles. Overall, these observations are consistent with previous findings

from human (Stuart *et al.* 2013) and animal (Denies *et al.* 2014) studies, showing that MetS condition, in particular insulin resistance, correlates with a higher content of glycolytic fibers and with a loss of the oxidative ones. In agreement with all these muscle structural changes, HFD animals showed a reduced physical performance in response to endurance training. Indeed, the HFD-induced new asset of muscle composition may account for the impairment of exercise performance, since the muscular fatigue resistance during endurance training exercises is based on the integrity and activity of slow type I/oxidative fibers. Interestingly, we found that the reduction of muscular oxidative metabolism was exalted by physical exercise with a further shift to a glycolytic phenotype, thus suggesting the incapability of HFD muscle of working through oxidative fibers that instead should be normally induced by the endurance training (Pette 2002, Shen *et al.* 2018).

In this regard, our results obtained in the testosterone-treated HFD group are of particular relevance for two reasons: (1) they demonstrate that testosterone was able to counteract all the HFD-induced changes in the skeletal muscle fiber composition of untrained rabbits, maintaining a glycolytic/oxidative fiber ratio similar to that of RD group; (2) they suggest that the maintenance of a 'RD-like' skeletal muscle composition by testosterone treatment allowed a normalization in the response to physical activity.

It is well known that testosterone exerts trophic effects on muscle, mainly promoting protein synthesis, fiber hypertrophy and satellite cells/myonuclei proliferation and differentiation (Herbst & Bhasin 2004). In fact, TTh is regarded an effective approach to increase muscle mass and strength in both young and old men with androgen deficiency (Bhasin *et al.* 1997, Bhasin *et al.* 2018). By using a murine model of aging-associated sarcopenia, TTh significantly enhanced muscle growth, while increasing muscle mass and hypertrophy of both types I and II fibers and preventing age-related increase in oxidative stress and muscle cell apoptosis (Kovacheva *et al.* 2010).

Moreover, TTh also reduced orchietomy-induced expression of several atrophy-related markers in skeletal muscle (White *et al.* 2013). In line with these anabolic and anti-catabolic effects of TTh, present data show a positive effect of testosterone on HFD-induced atrophy and on the expression of myogenic (MYOG, MYF5, PAX7) and differentiation (MYOD and ITGA7) genes that was also positively associated with testosterone plasma levels. Accordingly, an increase of PAX7 immunopositivity and the presence of newly generated MYH3-positive fibers were detected in skeletal muscle from testosterone-treated HFD rabbits. Noteworthy, we here demonstrate for the first time that testosterone normalized the MetS-induced shift of type I/II fibers, as demonstrated by PAS staining and MYH/Tbx15 gene expression, thereby promoting a muscle asset indicative of predominant oxidative metabolism, even in untrained rabbits. Accordingly, we found that the expression of mitochondrial enzymes crucial for cell respiration was not only significantly increased in skeletal muscle from testosterone-treated HFD rabbits, but also positively correlated to testosterone plasma levels. Moreover, ultrastructural analysis of muscle samples showed that testosterone was able to maintain mitochondrial integrity counteracting HFD-induced degeneration of internal membrane cristae. Similar results were found also in terms of both ATP production, as marker of mitochondrial respiration, and in terms of mitochondrial mass, as evaluated by TOM20 protein expression. Noteworthy, mitochondrial dysfunction is crucially contributing to the complex etiology of sarcopenia even in the context of metabolic diseases (Coen *et al.* 2018). Treatment in male mice increases the expression of genes involved in mitochondrial biogenesis and heat production in skeletal muscle *via* an AR-dependent pathway (Usui *et al.* 2014). In addition, in men, testosterone correlates with genetic and functional markers of mitochondrial function in skeletal muscle (Pitteloud *et al.* 2005). Therefore, the positive effects of testosterone treatment in MetS animals on fatigue resistant fibers and mitochondrial integrity – substantiated by a

**Table 5** Effects of physical exercise in testosterone-treated RD rabbits.

	RD + testosterone + PhyEx (n = 4)	p vs RD + PhyEx
Running distance (m)	204.00 (189.30; 218.70)	0.839
Running time (s)	612.00 (567.90; 656.10)	0.839
Lactate (nmol/L)	Pre-exercise: 2.70 (0.86; 4.54) End: 17.2 (2.87; 31.53)	0.374 0.839
Lactate/running distance (nmol/L for each 100 m)	8.59 (0.95; 16.23)	0.8393

Measurements were performed before starting (pre-exercise) and at the end of the exercise (end). The *P* value is vs the relative RD group of Table 3, untreated with testosterone. Data are reported as median with 95% confidence intervals (in brackets).



**Table 6** Metabolic parameters of testosterone-treated RD rabbits with and without PhyEx.

	RD + testosterone (n = 4)	P vs RD	RD + testosterone + PhyEx (n = 4)	P vs RD + PhyEx
Total body weight (kg)	3.75 (3.63–4.18)	0.885	3.72 (3.59–3.84)	0.454
Blood glucose (g/L)	0.97 (0.86–1.08)	0.262	0.93 (0.81–0.98)	0.304
OGTT (iAUC)	141.70 (130.12–176.07)	0.307	147.50 (137.67–155.30)	0.304
Cholesterol (mg/dL)	49.00 (48.25–49.75)	0.665	49.50 (48.25–50.00)	0.773
Triglycerides (mg/dL)	124.00 (88.50–149.00)	0.062	75.00 (67.50–87.00)	0.142
MAP (mmHg)	78.43 (65.00–96.09)	0.810	84.00 (80.00–88.00)	0.414
VAT (g/kg of total weight)	8.98 (7.43–9.85)	0.961	8.50 (7.66–9.35)	0.454
Testosterone (nmol/L)	51.20 (39.18–60.40)	0.001	71.60 (55.40–93.50)	0.003
LH (ng/mL)	0.05 (0.02–0.09)	0.014	0.06 (0.02–0.07)	0.014

Results are reported as medians with quartiles in brackets. The p value is vs the relative group of Table 1, untreated with testosterone.

significant improvement in the exercise performance and by the reduced lactate production – might be one of the major underlying mechanisms of TTh-induced metabolic improvements.

Altered glucose and lipid metabolism in HFD rabbits could thus be the cause of skeletal muscle modifications. Hence, we speculate that the amelioration of some MetS parameters (in particular fasting glucose levels, glucose tolerance and visceral adipose tissue accumulation) upon testosterone treatment could indirectly contribute to the normalization and/or improvement in skeletal muscle feature. Consistent with this hypothesis, we also found that TTh counteracted HFD-induced i.m. lipid accumulation. Recent studies demonstrated that a western-like diet may induce changes in skeletal muscle metabolism that promote lipid utilization over glucose oxidation as substrates of mitochondrial respiration (McCurdy *et al.* 2016, Gonzalez-Armenta *et al.* 2019). These changes were associated to a decrease in oxidative capacity and an impairment of glucose metabolism. Moreover, measures of mitochondrial respiration in response to fatty acids were significantly and positively correlated with insulin resistance (Gonzalez-Armenta *et al.* 2019) We did not analyze specific effects of HFD, with and without testosterone administration, on mitochondrial bioenergetics. However, our results, concerning HFD-induced and testosterone-counteracted skeletal muscle changes – including mitochondrial structural alterations, lipid accumulation, reduced content of oxidative fibers, as well as reduced oxidative metabolism – suggest that all these changes are associated to the diet composition. In addition, our results indicate that testosterone treatment counteracted these HFD-induced muscle changes, most likely facilitating muscle glucose uptake and oxidative metabolism, and this could be related to the maintenance of an intact mitochondrial structure.

In physiological conditions, blood glucose, plasma-free fatty acids, muscle glycogen, and i.m. triglycerides

are the major substrate sources for energy production in skeletal muscles. In particular, during exercise, blood glucose level can be increased primarily by means of an augmented glycogenolysis in the liver (Brooks *et al.* 2005). On the contrary, impairment of blood glucose homeostasis under MetS condition is associated with insulin resistance and altered glucose uptake by skeletal muscle. Accordingly, in RD+PhyEx, at the end of exercise, glycemia tended to increase, whereas in HFD+PhyEx rabbits the high glycemic levels remained unchanged. TTh partially reduced basal glycemia and allowed a sustained increase in glycemia upon PhyEx, thus suggesting a restoration of the glucose mobilization as important source of substrate for exercising muscle. However, based on our results we cannot rule out that metabolic improvements following TTh may be a consequence of positive direct effects of testosterone on skeletal muscle ability in metabolizing glucose and lipids. In this regard, we observed an up-regulation of IRS1 gene in the vastus medialis of testosterone-treated rabbits, in line with a previous study that obtained the same results on castrated rats treated with testosterone (Wu *et al.* 2010). Similarly, we report a positive effect of testosterone on the expression of genes involved in regulating lipid metabolism in skeletal muscle, such as PPAR $\alpha$ , LPL and UCPs. In human skeletal muscle, expression of PPAR $\alpha$  and of other genes regulating lipid metabolism, in particular LPL, are tightly related (Zhang *et al.* 2004). LPL activity, especially in oxidative muscle, is normally high to allow the release of free fatty acids and their subsequent uptake by myofibers, for energy production. UCPs are proteins located in the mitochondrial inner membrane with the function to uncouple the oxidation of fuels, mainly fatty acids, from ATP production, so the energy associated with oxidation is dissipated as heat (Bouillaud *et al.* 2016). Mitochondrial uncoupling and the consequent energy dissipation are of great importance in relation

to obesity, diabetes, and MetS (Li *et al.* 2018). UCP1 is highly expressed in brown adipose tissue with thermogenic function. However, an ectopic expression of UCP1 in the skeletal muscle of transgenic mice is associated with an increase of whole-body energy expenditure, resulting in the restoration of insulinemia, cholesterolemia and adiposity, and a decrease of type IIB fiber content (Li *et al.* 2000, Couplan *et al.* 2002). Moreover, UCP1 expression in the skeletal muscle of transgenic mice protects them from HFD-induced obesity-associated disturbances of glucose homeostasis (Katterle *et al.* 2008). UCP2 and UCP3 are broadly expressed UCP isoforms and their expression is upregulated after starvation and correlates with the use of fatty acids as mitochondrial substrates, suggesting a novel role, different from the uncoupling one (Bouillaud *et al.* 2016).

Generation of genetically modified knock-out (KO) mouse models in which the androgen receptor (AR) has been deleted either globally or in a tissue- and/or cell-specific manner gave also important insights into the mechanism by which androgens act on the musculoskeletal system. In global-ARKOs muscle mass was decreased by up to 20% compared with wild-type males (MacLean *et al.* 2008, Ophoff *et al.* 2009). However, in contrast with our observations, ARKO mice showed less fast-twitch (type II) and reduced contractile strength accompanied by an increase in the content of slow-twitch (type I) fibers (MacLean *et al.* 2008, Ophoff *et al.* 2009). The discrepancy indicates that the metabolic background may interfere with the testosterone actions on skeletal muscles as expected only considering the classical AR-mediated pathway. Moreover, data from several muscle-specific ARKO, including those targeting satellite cells, myocytes, myoblasts, and/or myofibers, provide evidence for a partial direct role of androgens *via* the AR in skeletal muscle to regulate its mass (Chambon *et al.* 2010, Rana *et al.* 2016). So it seems that other sites of action of testosterone might contribute in mediating the anabolic effects of testosterone on skeletal muscle mass. Indeed, a neuron-ARKO mouse model in which the AR is deleted specifically in neurons in the brain clearly demonstrated that brain AR acts to positively regulate hind-limb mass (Davey *et al.* 2017).

The most intriguing finding of our study is the demonstration that treating HFD rabbits with testosterone was able to allow a normal exercise capacity that was impaired before. This is tantamount to say that a testosterone supplementation might help MetS subjects to be able to perform physical exercise. Interestingly, when we treated with testosterone an additional group

of RD rabbits with normal level of testosterone, we did not observe any significant effects either on muscle fiber composition and metabolism or in terms of response to physical activity. We only evidenced a trophic effect, as demonstrated by an increased fiber diameter, as well as by an induction of some genes related to myogenesis, including PAX7 and ITGA7. Accordingly, testosterone plasma levels was positively associated with fiber diameter and ITGA7 mRNA expression in RD group, further supporting the view of a trophic effect of testosterone independently of HFD. Consistent with our findings, it has been demonstrated that testosterone administration in supraphysiological doses to healthy eugonadal men is associated with significant increases in muscle size due to muscle fiber hypertrophy without changing the relative proportions of type I and type II fibers (Sinha-Hikim *et al.* 2002). Overall, testosterone treatment in RD rabbits did not change the global metabolic conditions and affected the skeletal muscle features at a little extent, if compared to that observed in testosterone-treated HFD group. Hence, we can hypothesize that the positive effects of testosterone observed in the HFD group are linked to HFD-induced features, including testosterone deficiency, and not a mere androgenic effect, thus further supporting the use of TTh in pathological conditions characterized by hypogonadism but not in eugonadal subjects.

A recent meta-analysis of intervention studies in hypogonadal patients with metabolic derangements reported that combination of testosterone supplementation and lifestyle modifications was able to improve, more than lifestyle change itself, body composition (reduction of fat mass and increase in lean mass), leading to an amelioration of lipid profile and insulin resistance (Corona *et al.* 2015). Whether or not TTh could ameliorate exercise performance in MetS remains to be investigated. In fact, the available studies on the effect of testosterone supplementation on muscle strength and function were mainly conducted in older men and results are controversial. In a RCT, which compared older healthy men with low-to-normal testosterone levels receiving TTh or placebo for 3 years, a modest but significant improvement in muscle mass and power was shown in the active arm (Storer *et al.* 2017). Accordingly, in the 'Testosterone Trials', a coordinated set of seven placebo-controlled, double-blind trials aimed at determining the efficacy of increasing testosterone levels of older men with androgen deficiency, a small benefit was reported with respect to walking performance in hypogonadal patients following 12 weeks of testosterone therapy (Snyder *et al.* 2018).

However, this improvement was not confirmed when only older hypogonadal men with limited mobility were considered (Snyder *et al.* 2016). Conversely, in a trial involving more than 200 elderly hypogonadal men with limitations in mobility randomly treated with TTh or placebo for 9 months, a significant improvement in leg- and chest-press strength as well as in stair-climbing power was associated with TTh (Basaria *et al.* 2010). A recent reanalysis of systematic reviews and meta-analyses on the topic suggests that, in older men with clinical muscle weakness and low testosterone levels, treatment can be justified in daily clinical practice to improve muscle mass, muscle strength and/or physical performance (De Spiegeleer *et al.* 2018).

In conclusion, in an animal model of MetS, associated with hypogonadism, TTh showed a positive effect not only in ameliorating the glycometabolic profile, but also in modifying the morpho-functional features of skeletal muscles. Indeed, following testosterone treatment, HFD muscles were almost comparable to RD muscles. In fact, fiber muscle composition, mitochondria features and SDH activity were all improved and shifted toward an oxidative metabolism, instead of a glycolytic one. All the observed muscle modifications resulted in a better exercise performance. Accordingly, in aged male mice, testosterone administration, along with a low-intensity physical training, improved skeletal muscle mitochondrial biogenesis/quality and physical performance (Guo *et al.* 2012). Adequate clinical studies are required to confirm also in humans results obtained in these animal models. Our findings support the use of TTh in MetS as an intervention to favor lifestyle correction and, in particular, the adherence to physical exercise programs. As soon as lifestyle changes are established and could orchestrate their favorable effects (Morelli *et al.* 2019) the need for further testosterone supplementation can be reconsidered. Finally, our data do not suggest the use of testosterone administration to otherwise eugonadal individuals to improve aerobic endurance performances.

#### Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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