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Fiber type-specific hypertrophy and increased capillarization in skeletal muscle following testosterone administration of young women

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Short title: Testosterone provision and muscle morphology in young women

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25 ABSTRACT

26 It is well established that testosterone administration induces muscle fiber hypertrophy and
27 myonuclear addition in men, however, it remains to be determined whether similar
28 morphological adaptations can be achieved in women. The aim of the present study was
29 therefore to investigate whether exogenously administered testosterone alters muscle fiber
30 morphology in skeletal muscle of young healthy, physically active women. Thirty-five young
31 (20–35 years), recreationally trained women were randomly assigned to either 10-week
32 testosterone administration (10 mg daily) or placebo. Before and after the intervention,
33 hormone concentrations and body composition were assessed, and muscle biopsies were
34 obtained from the *vastus lateralis*. Fiber type composition, fiber size, satellite cell- and
35 myonuclei content, as well as muscle capillarization were assessed in a fiber type-specific
36 manner using immunohistochemistry. Following the intervention, testosterone administration
37 elevated serum testosterone concentration (5.1-fold increase, $P=0.001$), and induced
38 significant accretion of total lean mass (+1.9%, $P=0.002$) and leg lean mass (+2.4%,
39 $P=0.001$). On the muscle fiber level, testosterone increased mixed fiber cross-sectional area
40 (+8.2%, $P=0.001$), an effect primarily driven by increases in type II fiber size (9.2%,
41 $P=0.006$). Whereas myonuclei content remained unchanged, a numerical increase (+30.8%)
42 was found for satellite cells associated with type II fibers in the Testosterone group. In
43 parallel with fiber hypertrophy, testosterone significantly increased capillary contacts (+7.5%,
44 $P=0.015$) and capillary-to-fiber ratio (+9.2%, $P=0.001$) in type II muscle fibers. The current
45 study provides novel insight into fiber type-specific adaptations present already after 10
46 weeks of only moderately elevated testosterone levels in women.

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48 **KEYWORDS:** androgens, capillarization, myonuclei, myonuclear domain, satellite cells

49

50 NEW & NOTEWORTHY

51 We have recently demonstrated performance-enhancing effects of moderately elevated
52 testosterone concentrations in young women. Here we present novel evidence that
53 testosterone alters muscle morphology in these women, resulting in type II fiber hypertrophy
54 and improved capillarization. Our findings suggest that low doses of testosterone potentially
55 impacts skeletal muscle after only ten weeks. These data provide unique insights into muscle
56 adaptation and supports the performance-enhancing role of testosterone in women on the
57 muscle fiber level.

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74 INTRODUCTION

75 Androgens like testosterone are responsible for the development of secondary sex
76 characteristics and for maintaining tissue anabolism by stimulating cell growth and
77 differentiation through an androgen-receptor mediated pathway. Sex-based differences in
78 muscle mass are largely due to higher systemic testosterone levels in men (8–29 nmol L⁻¹),
79 compared to women (0.1–1.8 nmol L⁻¹) (26). When administered exogenously to men,
80 testosterone exerts anabolic actions on bone (57), hemoglobin (6) and skeletal muscle (5, 21),
81 thereby contributing to improved athletic performance. In women on the other hand, the
82 ergogenic effects are less well characterized (33), although, it is suggested that endogenous
83 androgen levels are associated with increased performance and muscle mass in competitive
84 athletes (4, 16). In line with this, we have recently demonstrated a causal effect of moderately
85 elevated testosterone concentrations (5-fold increase) on running time to exhaustion (+8.5%)
86 in young women (30).

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88 Short-term testosterone administration in men induces muscle fiber hypertrophy in a dose-
89 dependent manner (50, 51), and athletes on long-term anabolic steroid use display greater
90 muscle fiber cross-sectional areas (fCSA) compared to non-users (19, 37). Improvements in
91 size and contractile properties have also been identified down to the level of individual
92 muscle fibers (22). Intriguingly, slow type I fibers are reportedly more responsive to androgen
93 action than fast type II fibers (1, 19, 50). This argues for the possibility that type I and type II
94 fibers respond differently to increased testosterone concentrations. However, since these
95 findings are related to men, it remains to be determined whether testosterone administration
96 alters muscle fiber morphology also in young women, and whether these changes are fiber-
97 type dependent.

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One of the purported mechanisms by which testosterone regulates muscle growth is through increased myogenic activity, such as the activation, proliferation and subsequent fusion of muscle satellite cells into new myonuclei. In this respect, fiber hypertrophy in response to testosterone is associated with satellite cell-mediated myonuclear addition (51, 52). Similarly, long-term use of anabolic steroids has been shown to increase myonuclei content (19, 37), likely as a molecular mechanism to sustain protein synthesis during robust hypertrophy. Further evidence that androgens are critical for myogenic adaptation is found in men with pharmacologically suppressed testosterone levels, exhibiting a blunted myonuclear addition in response to resistance training (40).

Improvements in muscle performance following testosterone exposure may be underpinned by remodeling of the microvascular network. In addition to supplying muscle tissue with oxygenated blood and nutrients, recent advancements have revealed a key role for muscle capillaries in mediating hypertrophic adaptations (45, 55), likely through activation of the satellite cell pool (47, 48, 54). While it is commonly demonstrated that various forms of exercise increase capillary content (10, 29, 31), the effects of exogenous testosterone on muscle capillarization remains poorly understood, particularly in women.

Much work has been devoted to study the effects of testosterone in men, whereas, to the best of our knowledge, no study has yet addressed responses on the muscle fiber level in women. Improved body composition and muscle strength have previously been reported after testosterone replacement in postmenopausal women and in women with hypopituitarism (34, 44), however, it is challenging to apply these data to healthy young women increasing their testosterone concentration above normal physiological range. Therefore, in the present study, we sought to expand on our previous findings (30), and investigate whether the improvements

in physical performance were mediated by changes in muscle morphology. Moreover, we aimed to investigate the effects of testosterone on muscle fiber characteristics in women, with specific implications for the controversy in women's sports regarding eligibility regulations in athletes with naturally high testosterone levels. For ethical reasons, female elite athletes could not be investigated and therefore we chose young physically active women as our study population. Using immunohistochemistry, we aimed to determine fiber type-specific responses to testosterone in skeletal muscle of these women. Based on prior studies, we hypothesized that elevated testosterone levels in women would induce muscle hypertrophy, primarily in type I fibers, increase satellite cell- and myonuclei content and concomitantly expand the microvascular network.

METHODS

Ethical approval

All women were informed about the potential risks associated with the study after which they gave written consent. The study was approved by the local ethics committee in Stockholm (2016/1485-32, amendment 2017/779-32) and was carried out in accordance with the Declaration of Helsinki. The study is part of a larger project investigating the impact of testosterone administration on athletic performance/muscle mass in women (30). This trial was registered at ClinicalTrials.gov (NCT03210558).

Subjects

Thirty-five healthy young women (age: 28 ± 4 years) were included in the present study. Subjects were considered recreationally trained, regularly engaged in physical activities and performed sports or exercise at least 3 times/week, including both resistance- and endurance type exercise. Baseline characteristics of the subjects are presented in Table 1. Exclusion criteria were the presence of any medical disorder, oligomenorrhea or amenorrhea and intake of hormonal contraceptives during the last two months prior to the study. All women underwent gynecological examinations prior to the study, including ultrasound examinations of the uterus and ovaries.

Study design

The study is a randomized, double-blind, placebo-controlled study (RCT) in which the investigators, research coordinators, and the participating women were blinded to treatment allocation. Following screening, subjects were randomly assigned to either testosterone cream (10 mg, Andro-Feme® 1) or placebo cream, applied every evening to the outer thigh for 10 weeks. Based on previous studies (24), and the intention to increase testosterone levels about two-fold, the administered dosage (10 mg daily) was chosen to induce significant increases in systemic testosterone concentrations without causing severe harmful side effects. Baseline

data collection was performed in the early follicular phase of the menstrual cycle (cycle day 1-7) and final data collection at the end of the 10-week intervention period, without specific regards to phases of the menstrual cycle. Compliance and adverse events were monitored during the study and have been reported elsewhere (30). In addition, subjects were instructed to maintain their habitual training during the intervention. This was controlled by training records in which the type and amount of training was documented before (week 0) and in the end of the intervention (week 10). At both Baseline and end of treatment (Exit), body composition and strength were determined, and muscle biopsies and blood samples were collected.

Hormone concentrations

As described previously (30), serum concentration of testosterone was determined by liquid chromatography- tandem mass spectrometry (LC-MS/MS). In order to determine free androgen index, sex hormone-binding globulin (SHBG) was assessed by electrochemiluminescence immunoassay and free androgen index was calculated as testosterone nmol L⁻¹ divided by SHBG nmol L⁻¹ · 100.

Body composition

Body composition (total lean mass, leg lean mass and fat mass) was investigated at Baseline and Exit using dual energy X-ray absorptiometry (DXA), (Lunar Prodigy Advance, GE Healthcare, USA), previously described elsewhere (16).

Muscle strength

After a standardized warm up, subjects performed three right leg maximal voluntary isometric knee extensions at a 60 degree knee angle. After low pass filtering at 50Hz, the knee extensor torque signal (Isomed2000, D&R Ferstl GmbH, Henau, Germany) was sampled at 5000 Hz (Spike2, version 7.09, CED, England). Peak torque was calculated as the highest torque

attained during a 3s effort. Peak rate of torque development was calculated as the peak value of the derivative of the torque time curve.

Muscle biopsy sampling

Muscle biopsies at Baseline and Exit were collected approximately 60 minutes after the subjects had completed a set of performance tests (jump tests, knee extension torque, running time to exhaustion and Wingate anaerobic power test), as described earlier (30). Biopsies were taken from the middle portion of the *vastus lateralis* muscle approximately 15 cm above the patellae (at a depth of 2-3 cm). After administration of local anesthesia (Carbocaine 20 mg ml⁻¹, AstraZeneca AB, Sweden), a small incision was made to the skin and fascia and samples of 50-100 mg were obtained with a Weil-Blakesley conchotome (14). After quickly removing visible blood or connective tissue, fiber bundles were oriented perpendicular to the horizontal surface and mounted in O.C.T embedding medium (Tissue-Tek® O.C.T compound), thereafter quickly frozen in isopentane cooled by liquid nitrogen. These specimens were subsequently stored at -80°C until sectioning commenced.

Immunohistochemistry

Muscle cross-sections (7 µm) were cut at -21°C using a cryostat (Leica CM1950) and care was taken to align samples for subsequent cross-sectional analyses. Cryosections were then placed on microscope glass slides (VistaVision™, VWR International), allowed to air-dry for 60 min and stored at -80°C. Samples from Baseline and Exit were mounted together to minimize variability in staining efficiency.

For muscle fiber type composition, unfixed slides were incubated overnight with a primary antibody against laminin (1:50; D18, Developmental Studies Hybridoma Bank (DSHB), USA) in order to delineate fiber borders. The following day slides were incubated for 60 min with primary antibodies against myosin heavy chain (MHC) isoform proteins; MHC-I (1:500; BA-F8, DSHB, USA) and MHC-II (1:250; SC-71, DSHB, USA), diluted in PBS (1% normal

goat serum and 1% fat-free dry milk). After washes in Phosphate buffered saline (PBS), species- and subclass specific secondary fluorescent antibodies were applied for 60 min (1:100; Goat anti-mouse 350 IgG_{2A}, 1:500; 488 Goat anti-mouse IgG_{2B} and 1:500; 594 Goat anti-mouse IgG₁, Alexa Fluor, Invitrogen, USA). Slides were then mounted with cover glasses using Prolong Gold Antifade Reagent (Invitrogen, USA). This staining rendered blue fiber borders and enabled differentiation between type I (green) and type II muscle fibers (red), as depicted in Figure 3.

For fiber type-specific satellite cell staining, slides were fixed in 4% paraformaldehyde (PFA) for 20 min, washed in PBS and blocked for 30 min in 1% bovine serum albumin and 0.01% of Triton X-100. Thereafter, incubated overnight with a cocktail of antibodies against laminin (1:200; D18, DSHB, USA), MHC-I (1:500; BA-F8, DSHB, USA), and Pax7 (1:100; 199010, Abcam, GBR), diluted in 1% normal goat serum. The next day, slides were incubated with secondary antibodies (1:500; 488 Goat anti-mouse IgG_{2A}, 1:2000; 488 Goat anti-mouse IgG_{2B} and 1:500; 594 Goat anti-mouse IgG₁, Alexa Fluor, Invitrogen, USA), prior to mounting with Prolong Gold Antifade Reagent containing 4',6-diamidino-2-phenylindole (DAPI, Invitrogen, USA) to permit labeling of nuclei. Satellite cells were visualized in red, nuclei in blue, laminin border and type I muscle fiber in green, where the latter being weakly stained to clearly separate fiber borders from type I fibers (Figure 4).

For fiber type-specific capillary staining, slides were fixed in 2% PFA for 5 min, washed in PBS and incubated for 2h at room temperature with antibodies against laminin (1:50; D18, DSHB, USA) MHC-I (1:500; BA-F8, DSHB, USA) and CD31/PECAM (1:400; JC70, Santa Cruz Biotechnology, USA). Following PBS washes, slides were incubated with secondary antibodies (1:500; 350 Goat anti-mouse IgG_{2A}, 1:1000; 488 Goat anti-mouse IgG_{2B}, 1:1000; 594 Goat anti-mouse IgG₁, Alexa Fluor, Invitrogen, USA), diluted in PBS (1% normal goat serum), before being mounted with Prolong Gold Antifade Reagent (Invitrogen, USA). These

procedures stained capillaries in red, laminin border in blue and type I muscle fibers in green, depicted in Figure 5.

Image acquisition and analysis

Stained sections were visualized on a computer screen connected to a widefield fluorescent microscope (Celena® S, Logos Biosystems, South Korea) and pictures were digitally captured and processed using image analysis software (Celena® S Digital Imaging System, Logos Biosystems, South Korea). Fluorescence signal was recorded using mCherry excitation filter (580/25 nm), EYFP excitation filter (500/20 nm) and DAPI excitation filter (375/28 nm). All morphological analyses and subsequent quantification were performed in ImageJ (National Institutes of Health, USA) by an experienced investigator blinded to the subject coding. For analyses of fCSA and fiber type composition, all biopsies were included (n=35), whereas five biopsies for Pax7-staining (Placebo=15, Testosterone=15) and two biopsies for CD31-staining (Placebo=15, Testosterone=18) were excluded due to poor tissue quality or insufficient number of fibers.

In MHC/laminin-stained slides, the number of fibers of each type was counted on the whole muscle section to accurately determine fiber type composition, including an average of 694 ± 326 (SD) fibers per biopsy (9). Their relative abundance was then expressed as percentage of total fiber number. From the same slides, 4-6 areas from different regions of the biopsy, free of freezing artefacts were used for assessment of fCSA. Within each area, fCSA was measured by manually encircling the laminin border and 50 fibers per fiber type were included for analysis (8, 43). These fibers were randomly selected but fibers oriented longitudinally or fibers located in the periphery of the biopsy were not considered for analysis. Mean fCSA was then obtained by averaging individual fiber sizes. The analysis of fiber size distribution was performed by distributing fibers into size intervals of $1000 \mu\text{m}^2$ to compare their relative frequency. The form factor was used to ensure fiber circularity in

muscle cross-sections; $(4\pi \cdot \text{fCSA}) / (\text{fiber perimeter})^2$ (10) and this did not differ between pre-post measurements (data not shown).

To determine fiber type-specific satellite cell abundance, MHC-I and laminin staining was applied in conjunction with the Pax7-antibody, previously used for satellite cell identification in human skeletal muscle (56). Pax7-positive cells (Pax7⁺) located inside the laminin border were, in case of colocalization with DAPI determined satellite cells and then marked together with their associated fiber type, either type I (MHC-I positive) or type II (MHC-I negative), see Figure 4 for example. An average of 348 ± 96 (SD) fibers per biopsy were included for satellite cell quantification and were then expressed as; number per fiber, in relation to myonuclei content (% satellite cells) and per fiber area (mm²). From the same staining, myonuclei content was determined separately for each fiber type. To counter one of the inherent difficulties analyzing myonuclei content using a widefield microscope, we consistently adhered to the criterion that nuclei had to have more than half of its geometrical center residing inside the basal lamina to be considered myonuclei (DAPI⁺/Pax7⁻). As suggested in previous reports (42), the analysis included 50 fibers/fiber type/biopsy and myonuclei content was expressed as; per fiber and in relation to fiber area, i.e. myonuclear domain.

Quantification of fiber type-specific capillary indices was performed in accordance with previous work (28), comprising capillary contacts (CC; number of capillaries in contact with each individual fiber) and the capillary-to-fiber ratio (C/F_i; capillaries in contact with each individual fiber considering their sharing factor). To further quantify muscle fiber diffusion capacity we also determined the individual capillary-to-fiber perimeter exchange ratio (CFPE; capillaries per 1000 μm⁻¹) as a quotient between C/F_i and fiber perimeter. Capillary indices were calculated separately for type I and type II fibers including 25 fibers/biopsy as a minimum.

Statistics

Data are presented as means \pm SD. Normality of the data was assessed with Shapiro-Wilk test and data were log-transformed in case of significance. Baseline comparisons were performed with unpaired student's t-test. Effect of intervention was analyzed using two-way mixed ANOVA with factors for group (Testosterone vs Placebo) and time (Baseline vs Exit). Holm-Sidak's multiple comparison was used as *Post hoc* test to localize the effects of each ANOVA model revealing significant interaction. Pearson product-moment correlation coefficient (r) was used to investigate the relationship between hormone concentrations and outcome measures. Analyses were completed using GraphPad Prism (version 8.0.0 for Windows, GraphPad Software, USA). Statistical significance was accepted at $P < 0.05$.

RESULTS

Subject characteristics

Subject characteristics are presented in Table 1. Except for body mass (main effect of group, $P < 0.05$), the groups were comparable at Baseline and no changes were observed over time.

Hormone concentrations

Hormone concentrations are presented in Table 1. The included participants had at Baseline an average testosterone concentration of 0.90 nmol L^{-1} (range from 0.30 to 1.63 nmol L^{-1}). As expected, the Testosterone group significantly increased testosterone levels to an average concentration of 4.65 nmol L^{-1} (interaction effect, $P < 0.01$; +5.1 fold; range from 1.48 to $12.75 \text{ nmol L}^{-1}$, $P=0.001$) and the free androgen index (interaction effect, $P < 0.01$; +6.5 fold; $P=0.001$), whilst no corresponding changes were observed in the Placebo group.

Training records

Training records collected before (week 0) and at the end of the intervention (week 10) were obtained from a limited number of subjects due to low reporting adherence (Placebo; $n=12$, Testosterone; $n=13$). Training habits were similar between groups and remained unchanged over time, see Table 1.

Body composition

No differences between groups were observed at Baseline for measures of body composition (Table 1). However, after the intervention, a tendency for interaction effect for total lean mass ($P=0.067$), and an interaction effect ($P < 0.05$) for leg lean mass was found, whereby the Testosterone group displayed significant increases in total lean mass (+1.9%, $P=0.002$) and leg lean mass (+2.4%, $P=0.001$). No changes were observed for fat mass (kg) and percent body fat in any of the groups.

342 *Muscle strength*

343 Isometric knee extension peak torque was similar between groups at Baseline and no changes
 344 were observed following the intervention (209 ± 10 to 211 ± 10 Nm vs 218 ± 8 to 224 ± 9 Nm
 345 in the Placebo and Testosterone group, respectively). Similarly, peak rate of torque
 346 development did not change (1213 ± 70 to 1154 ± 70 Nm/s vs 1264 ± 63 to 1214 ± 85 Nm/s
 347 in the Placebo and Testosterone group, respectively).

348 *Fiber type composition and fiber size*

349 Results for fiber type composition and fiber size are shown in Table 2. In response to the
 350 intervention, fiber type composition remained unchanged. However, for fCSA in mixed
 351 fibers, an interaction effect was found ($P < 0.05$), in which the Testosterone group displayed a
 352 significant increase ($+8.2\%$, $P=0.001$). When further analyzing the different fiber types, type I
 353 fCSA displayed a main effect of time ($P < 0.05$). For type II fCSA, an interaction effect was
 354 found, whereby the Testosterone group demonstrated a significant increase following the
 355 intervention ($+9.2\%$, $P=0.006$). The increase in type II fCSA could also be visualized by the
 356 size distribution analysis, demonstrating a rightward shift for type II fibers in the Testosterone
 357 group (Figure 1D).

358 *Fiber type- specific satellite cell- and myonuclei content*

359 Fiber type- specific satellite cell- and myonuclei content is shown in Figure 2. Type II fibers
 360 revealed a trend for main effect of time in satellite cells per fiber ($P=0.054$) and percent
 361 satellite cells ($P=0.079$). Although, no differences were observed between the groups (no
 362 interaction effect), the tendency for change over time for satellite cells in type II fibers
 363 seemed to be primarily driven by increases in the Testosterone group ($+30.8\%$, 12/15
 364 participants increased), and not by the Placebo group ($+3.6\%$, 6/15 participants increased).
 365 This was although not the case for percent satellite cells. Likewise, satellite cell content per

square millimeter (SCs per mm²) was not altered and both myonuclei per fiber and myonuclear domain remained constant (Figure 2).

Fiber type-specific capillary content

Muscle fiber type-specific capillary content is shown in Table 3. Following the intervention, type II CC and type I C/Fi displayed a main effect of time ($P < 0.05$). Again, the change over time for type II fiber CC (no interaction effect) seemed to be driven by the Testosterone group (Testosterone; +7.5%, 15/18 participants increased, Placebo; 2.2%, 9/18 participants increased). Furthermore, a trend for interaction ($P=0.060$) was found for type II fiber C/Fi, in which the Testosterone group displayed a significant increase (9.2%, $P=0.002$), compared to an unchanged Placebo group. However, type I and type II fiber CFPE remained unchanged in response to the intervention.

Correlations

No correlations were found between basal hormone concentrations (serum testosterone and free androgen index) and muscle morphology, body composition or strength. Likewise, changes in hormone concentrations in the Testosterone group were not associated to alterations in muscle morphology or body composition ($P > 0.05$).

DISCUSSION

The present study sought to determine the effects of exogenous testosterone on muscle fiber morphology in young women. The main finding observed here was that moderately elevated testosterone levels for 10 weeks (fivefold above normal physiological range) induced alterations in skeletal muscle morphology, manifested as type II fiber hypertrophy and an expansion of the microvascular network. Interestingly, the apparent fiber hypertrophy was not accompanied by addition of new myonuclei, and only numerical increases were observed for satellite cell content, suggesting that satellite cell-mediated myonuclear addition is of less importance during early stages of testosterone-induced hypertrophy. We hereby provide novel physiological insights in women receiving exogenous testosterone and our data largely conforms to the well-defined anabolic properties of testosterone in skeletal muscle.

It is consistently shown that testosterone administration in men increases strength and muscle mass (5, 59), and mitigates muscle wasting during ageing or clinical conditions (7, 20, 25).

Studies investigating whether testosterone exerts ergogenic or anabolic actions in women are nevertheless scarce. Besides perceived effects documented in women self-administering anabolic steroids (60), improvements in strength and body composition have been reported in androgen deficient women after testosterone replacement therapy (34, 44), though the latter study detected effects first after reaching high serum concentrations ($\approx 7 \text{ nmol L}^{-1}$). In accordance, but with lower systemic levels ($\approx 4.5 \text{ nmol L}^{-1}$), we demonstrate for the first time that exogenously administered testosterone improves muscle morphology in healthy euandrogenic women. It thus appears that testosterone induce ergogenic effects independently of sex and that effects in young women can be achieved at lower serum concentrations than in postmenopausal women. Interestingly, some studies employing dose-response designs have found correlations between increasing systemic testosterone levels and changes in muscle mass (6, 34). However, in the present study, no such correlations were found. Even though

blood sampling was standardized according to clock time and sampling method, this lack of association may potentially be explained by variations in absorption of testosterone, phase of menstrual cycle at Exit (15), or diurnal fluctuations. Furthermore, we found that testosterone induces alterations in body composition by increasing total and leg lean mass, which from a sport performance perspective might be advantageous to athletes engaged in strength- and power disciplines. This finding fits well with previous work on muscle protein turnover, showing that short-term testosterone administration increases protein synthesis in women (53, 64). It is therefore likely that the positive protein balance was driven by changes in rates of protein synthesis, considering its key role in determining protein turnover in healthy adults (12). It should be noted, however, that despite gains in muscle mass, no apparent transfer into improved muscle function could be demonstrated here. Lastly, in an attempt to separate the impact of testosterone on changes in lean mass, from those potentially confounded by behavioral alterations such as motivation, energy levels etc., we analyzed training records. Since training habits remained unchanged throughout, the effects can be ascribed to the testosterone administration *per se*, rather than a corresponding rise in training frequency.

To expand on previous data in women, we collected muscle biopsies in order to address adaptations occurring on the muscle fiber level. Consistent with observations following short-term testosterone administration in men (50), and long-term use of anabolic steroids (37), muscle fiber type composition was unaffected by testosterone. The concept that fiber type composition is not under hormonal regulation is further supported when comparing muscle biopsies from men and women, displaying equal relative proportion of fiber types (58). In rat muscle though, there is evidence of a shift towards a more glycolytic phenotype following testosterone exposure (32), but such findings have not yet been confirmed in human skeletal muscle. However, it should be considered that our analyses do not distinguish between type II muscle fiber subclasses. Thus, fiber type transition within type II fibers still remains a

possibility, such as those described after resistance training and periods of detraining (2). It is also plausible that the relatively short intervention period limited fiber type transitions.

In line with our hypothesis, testosterone induced muscle fiber hypertrophy, although unexpectedly, this was mainly driven by increases in type II fiber size. This observation somewhat contradicts prior findings in men, in which greater relative type I fiber hypertrophy has been observed in the vastus lateralis muscle (1, 19, 50), but not in the deltoid muscle (27). Intriguingly, some studies indicate that testosterone responsiveness is fiber type-dependent, by showing that lower doses of testosterone (300 mg per week) sufficiently provoke type I fiber hypertrophy, whereas higher doses (600 mg per week) were required to stimulate growth of type II fibers (50). In this respect, we believe that discrepancies between our findings and those in men could be due to differences in relative fiber size. Unlike women, men typically exhibit greater type II fCSA compared to type I (37, 58), raising the possibility that type II fibers are closer to their upper limit in terms of growth potential, thus making them less responsive to anabolic stimuli, e.g., testosterone administration. In support of this, women have type II fibers equal to, or smaller than their type I counterparts (58), suggesting a greater growth capacity in these fibers, which might explain the more marked hypertrophic response seen in type II fibers in our study. On the other hand, we cannot exclude that type I and type II fibers types may have distinct inherent properties affecting their responsiveness to androgens. While androgen receptors (AR) and the mTOR signaling pathway are recognized as central mediators of testosterone action (3, 36), it has been reported that AR content is greater in type I compared to type II fibers (35), indicative of a mechanism for fiber type-specific responses to testosterone in men. Likewise, key components of the mTOR pathway are differently expressed and phosphorylated in the two fiber types, both during rest and in response to contractile activity (13, 61). However, whether these factors regulate fiber type-specific adaptations in women differently provides a basis for further investigation.

In the present study, testosterone did not significantly modulate satellite cell- or myonuclei content, a finding that opposed our initial hypothesis. Even though a numerical increase was noted in the testosterone group for satellite cells associated with type II fibers, suggesting heightened myogenic activity (pre-post comparison), this did not reach statistical significance (interaction effect). While testosterone administration is traditionally associated with increased satellite cell abundance, this is based on men receiving supraphysiological doses (≥ 300 mg per week) for 20 weeks (51, 52). However, this is not observed after low or moderate doses. Thus, rather than being a sex-based disparity, we believe that conflicting findings are related to the administered dose, or to the length of intervention, or a combination thereof. Nevertheless, we cannot rule out that this occurrence is sex-related. Others have shown that women, in comparison to men, do not upregulate markers of satellite cell proliferation following transient increases in circulating testosterone (41). Instead, women seem to rely on estrogen-related signaling for maintenance (11) and for the activation of the satellite cell pool after contractile activity (18). Thus, the lack of satellite cell activity may be attributed to interference with endogenous estrogen signaling, albeit this remains speculative and requires further analyses.

Moreover, testosterone-induced hypertrophy is assumed to be directly reliant on satellite cell proliferation and subsequent addition of new myonuclei. However, in good agreement with our findings, this hypothesis was recently challenged using a rodent model of satellite cell ablation, in which comparable increases in muscle mass was found in satellite cell ablated animals and control animals after testosterone treatment (17). We therefore suggest that, at least up to a certain limit, satellite cell-mediated myonuclear addition does not appear critical for testosterone-induced hypertrophy. Our data also supports the notion that pre-existing myonuclei have a reserve capacity to sustain moderately elevated transcriptional demands. This was experimentally shown by Kirby et al. 2016, demonstrating an increase in both the

number of transcriptionally active myonuclei and the rate of transcription per myonucleus in the event of satellite cell ablation (39). In perspective of the myonuclear domain ceiling theory described in human muscle (38, 49), the magnitude of type II fiber growth here is below the theoretical threshold for when additional myonuclei are required to sustain further increases in fiber size. It thus appear that a substantial amount of fiber hypertrophy can be achieved without incorporating myonuclei. Intriguingly, this seems particularly true for type II fibers, exhibiting less rigid myonuclear domains than type I fibers (46), providing support for the findings of the present study.

Little is currently known regarding the effects of androgens on microvascular adaptations. Data in rat muscle is rather inconclusive (23, 32) and the evidence gathered from human studies is limited. Athletes using anabolic steroids exhibit greater CC compared to non-users, though differences disappeared when adjusted for fiber size (65). More relevant to the present study, young men receiving graded doses of testosterone (25-600 mg per week) did not improve muscle capillarization after 20 weeks (50). While we found supporting evidence that the microvascular network expanded specifically in type II fibers, discrepancies may be related to the lack of fiber type-specific analyses in previous studies, potentially masking subtle changes on an individual fiber basis.

Furthermore, we found that capillary indices in type I fibers remained constant, whereas increases were seen in type II fiber C/Fi and to a certain extent also CC (no interaction effect, but significant pre-post increase). Given the close relationship between CC and C/Fi, we argue that augmented C/Fi reflects capillaries in new positions, as described elsewhere (28), rather than a rearrangement of the existing capillary network, thereby supportive of increased CC in type II fibers. Intriguingly, in relation to the hypothesis that an increased fiber size may impair diffusion capacity (62), we addressed this issue by calculating capillary indices related to fiber perimeter. Our data show that regardless of fiber hypertrophy, capillary supply is

effectively maintained as CFPE remained unchanged. This is relevant from a performance perspective as improved type II fiber size and a preserved capacity for blood-tissue exchange might underlie the previously observed improvements in running time to exhaustion (30). Despite the fact that type II fiber capillarization improved after the intervention, it remains difficult to determine whether testosterone stimulated angiogenesis *per se*, or whether microvascular adaptations occur subsequently to fiber hypertrophy induced by testosterone. In this context, time course studies of muscle hypertrophy and angiogenesis have demonstrated that these seemingly distinct processes are tightly interconnected, as fiber hypertrophy occurs concomitantly with expansion of the microvasculature (31). The effect of testosterone on muscle capillarization identified here is therefore likely part of the muscle adaptive response to ensure adequate perfusion. Additionally, we acknowledge that our analyses are limited to capillaries running in parallel to muscle fibers and that we did not consider the length of capillary contacts, thus excluding potential inferences regarding micro vessel tortuosity.

Some limitations requires further consideration. First, muscle biopsies were collected after the subjects completed a session of physiological tests (within 60 minutes), potentially triggering adaptive responses within the tissue. Despite that the included measures are likely unaffected by prior activity, satellite cell content might be elevated shortly after exercise. However, such acute responses are shown specifically in response to high-volume eccentric protocols (63), thereby making an interference effect unlikely in the present study. This effect would also have been negligible as samples were obtained under the same conditions at both Baseline and Exit. Second, we acknowledge the rather subjective element of assessing myonuclei content in histological sections using a widefield microscope. Although this procedure still remains sensitive to some degree of operator subjectivity, we countered this by conducting morphological analyses in a blinded fashion while adhering to the pre-defined criteria for determining myonuclear location (described in the method section). Third, the lack of data

collection on appetite and dietary intakes may be considered a limitation as we cannot completely exclude the influence of these factors. Lastly, the fact that muscle biopsies were not obtained from all subjects could explain the lack of statistical significance in variables where robust numerical increases were observed. Despite these limitations, using a RCT design, we provide novel insights into the effects of testosterone on muscle morphology in women. Although, we did not investigate elite athletes but physically active women, we believe that these data are relevant from a women's sports perspective and may have important implications for the ongoing controversy regarding hyperandrogenic women in sports.

In conclusion, this study provides novel evidence that moderate doses of exogenous testosterone for 10 weeks induce lean mass accretion, type II muscle fiber hypertrophy and a concomitant expansion of the microvascular network in skeletal muscle of young healthy women. Furthermore, testosterone-induced muscle fiber hypertrophy does not seem to be reliant on myonuclear addition and only a modest expansion of the satellite cell pool was found after testosterone administration.

ADDITIONAL INFORMATION

Competing interests: The authors declare that they have no conflict of interests.

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Figure legends

Figure 1. Muscle fiber size distribution in the Placebo (A; type I fibers, B; type II fibers) and the Testosterone group (C; type I fibers, D; type II fibers) at Baseline (white bars) and at Exit (grey bars). Placebo (n=16) and the Testosterone group (n=19).

Figure 2. Fiber type-specific satellite cell- and myonuclei content at Baseline (white bars) and Exit (gray bars) in the Placebo (n=15) and the Testosterone group (n=15). Values are means \pm SD. (*) indicates tendency for main effect of time ($P=0.054$). (#) indicates tendency for main effect of time ($P=0.079$).

Figure 3. Representative images showing muscle fiber type composition (A) and fiber borders for measurement of fCSA (B) in muscle cross section. A, Merged image of MHC type I (green), MHC type II (red) and laminin (blue). B, Single channel laminin (blue). Scale bar = 50 μ m.

Figure 4. Representative images of fiber type-specific satellite cell staining (A-D) in muscle cross-section. A, Merged image of MHC type I (green), laminin (green), Pax7 (red), DAPI (blue). Arrowheads (white) indicate satellite cells. B, Single channel Pax7 (red). C, Merged image of MHC type I (green and laminin (green). D), Single channel DAPI (blue). Scale bar = 50 μ m.

Figure 5. Representative images of fiber type-specific capillary staining (A-B) in muscle cross-section. A, Merged image of MHC type I (green), laminin (blue), CD31 (red). B, Single channel CD31 (red). Arrowheads (white) indicate muscle capillaries. Scale bar = 50 μ m.

Table 1. Subject characteristics, hormone concentrations, training records and body composition at Baseline and Exit in the Placebo and Testosterone group.

	Placebo		Testosterone	
	Baseline	Exit	Baseline	Exit
<i>Subject characteristics</i>				
<i>n</i>	16	-	19	-
Age (y)	27.4 ± 4.1	-	27.7 ± 3.1	-
Height (cm)	166.9 ± 6.2	-	170.2 ± 4.8	-
Body mass (kg)	63.4 ± 7.3	63.7 ± 7.4	68.3 ± 6.5‡	68.5 ± 6.7‡
BMI (kg m ⁻²)	22.7 ± 1.9	22.8 ± 1.9	23.6 ± 1.6	23.7 ± 1.9
<i>Hormone concentrations</i>				
Testosterone (nmol L ⁻¹)	0.89 ± 0.21	1.05 ± 0.37	0.91 ± 0.37	4.65 ± 2.96* [#]
Free androgen index	1.19 ± 0.41	1.23 ± 0.48	1.28 ± 0.53	8.32 ± 5.41* [#]
<i>Training (per week)</i>				
Total sessions	6.0 ± 2.0	5.8 ± 3.5	7.3 ± 2.3	6.8 ± 1.9
Endurance exercise	2.2 ± 2.4	2.4 ± 2.6	2.7 ± 1.4	2.4 ± 1.5
Resistance exercise	1.9 ± 1.6	2.0 ± 1.7	2.2 ± 2.0	3.0 ± 2.8
Other	1.9 ± 1.9	1.4 ± 1.8	2.4 ± 1.7	1.3 ± 1.7
<i>Body composition</i>				
Total lean mass (kg)	44.8 ± 5.6	45.0 ± 5.8	47.4 ± 4.5	48.3 ± 4.6*
Leg lean mass (kg)	15.0 ± 1.9	15.1 ± 2.0	16.1 ± 1.5	16.5 ± 1.6* [#]
Fat mass (kg)	16.0 ± 4.2	16.1 ± 4.8	18.1 ± 6.1	17.3 ± 6.1
Body fat (%)	26.2 ± 5.6	26.2 ± 6.1	27.3 ± 7.6	26.0 ± 7.6

Values are means ± SD. BMI, body mass index. n=35 for all data except training records (n=25;

Placebo; n=12, Testosterone; n=13). ‡ indicates significant main effect of group (P < 0.05).

significantly different from Placebo (P < 0.05). *significantly different from Baseline (P < 0.05).

Table 2. Muscle fiber type composition and fiber size at Baseline and Exit in the Placebo and Testosterone group.

	Baseline	Placebo Exit	Δ	Baseline	Testosterone Exit	Δ
<i>Fiber type composition %</i>						
Type I	45.4 \pm 9.0	44.9 \pm 8.6	-0.7 \pm 6.0	48.9 \pm 9.3	50.0 \pm 8.3	+1.07 \pm 4.8
Type II	54.6 \pm 9.0	55.1 \pm 8.6	+0.7 \pm 6.0	51.1 \pm 9.3	50.0 \pm 8.3	-1.07 \pm 4.8
<i>fCSA (μm^2)</i>						
Mixed fibers	4619 \pm 778	4700 \pm 617	+81 \pm 318	4844 \pm 933	5240 \pm 922*	+395 \pm 510
Type I	4661 \pm 738	4804 \pm 599 [†]	+143 \pm 309	4753 \pm 822	5131 \pm 794 [†]	+379 \pm 534
Type II	4572 \pm 930	4589 \pm 748	+17 \pm 352	4952 \pm 1168	5407 \pm 1189* [#]	+455 \pm 584

Values are means \pm SD. fCSA, fiber cross-sectional area. Δ , change from pre-post intervention, [†] indicates significant main effect of time ($P < 0.05$). *significantly different from Baseline ($P < 0.05$).
[#] significantly different from Placebo ($P < 0.05$).

Table 3. Muscle fiber type-specific capillary content at Baseline and Exit in the Placebo and Testosterone group.

	Baseline	Placebo Exit	Δ	Baseline	Testosterone Exit	Δ
<i>CC</i>						
Type I	4.42 \pm 0.84	4.46 \pm 0.80	+0.04 \pm 0.38	4.52 \pm 0.61	4.64 \pm 0.56	+0.12 \pm 0.40
Type II	3.99 \pm 0.92	4.08 \pm 0.83 [†]	+0.09 \pm 0.40	4.12 \pm 0.77	4.43 \pm 0.48 [†]	+0.31 \pm 0.51
<i>C/Fi</i>						
Type I	1.64 \pm 0.30	1.70 \pm 0.34 [†]	+0.06 \pm 0.12	1.69 \pm 0.26	1.74 \pm 0.26 [†]	+0.06 \pm 0.20
Type II	1.48 \pm 0.34	1.52 \pm 0.36	+0.04 \pm 0.13	1.52 \pm 0.28	1.66 \pm 0.20*	+0.14 \pm 0.19
<i>CFPE</i>						
Type I	6.37 \pm 0.85	6.23 \pm 0.97	-0.14 \pm 0.65	6.32 \pm 0.50	6.29 \pm 0.57	-0.03 \pm 0.37
Type II	5.75 \pm 0.95	5.67 \pm 0.94	-0.08 \pm 0.67	5.65 \pm 0.59	5.89 \pm 0.56	+0.24 \pm 0.57

Values are means \pm SD. CC, capillary contacts, C/Fi, capillary-to-fiber ratio, CFPE, capillary-to-fiber perimeter exchange ratio. Placebo (n=15) and the Testosterone group (n=18). Δ , change from pre-post intervention, [†] indicates significant main effect of time ($P < 0.05$). *significantly different from Baseline ($P < 0.05$).

Figure 1.

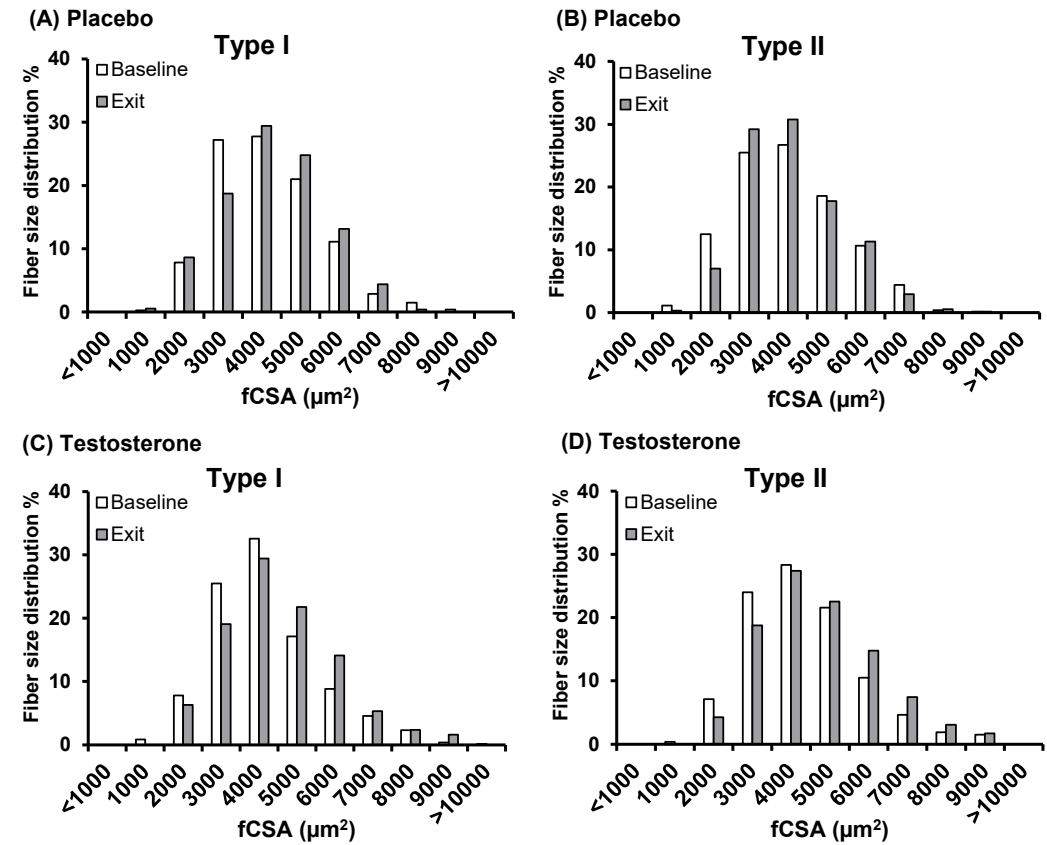


Figure 2.

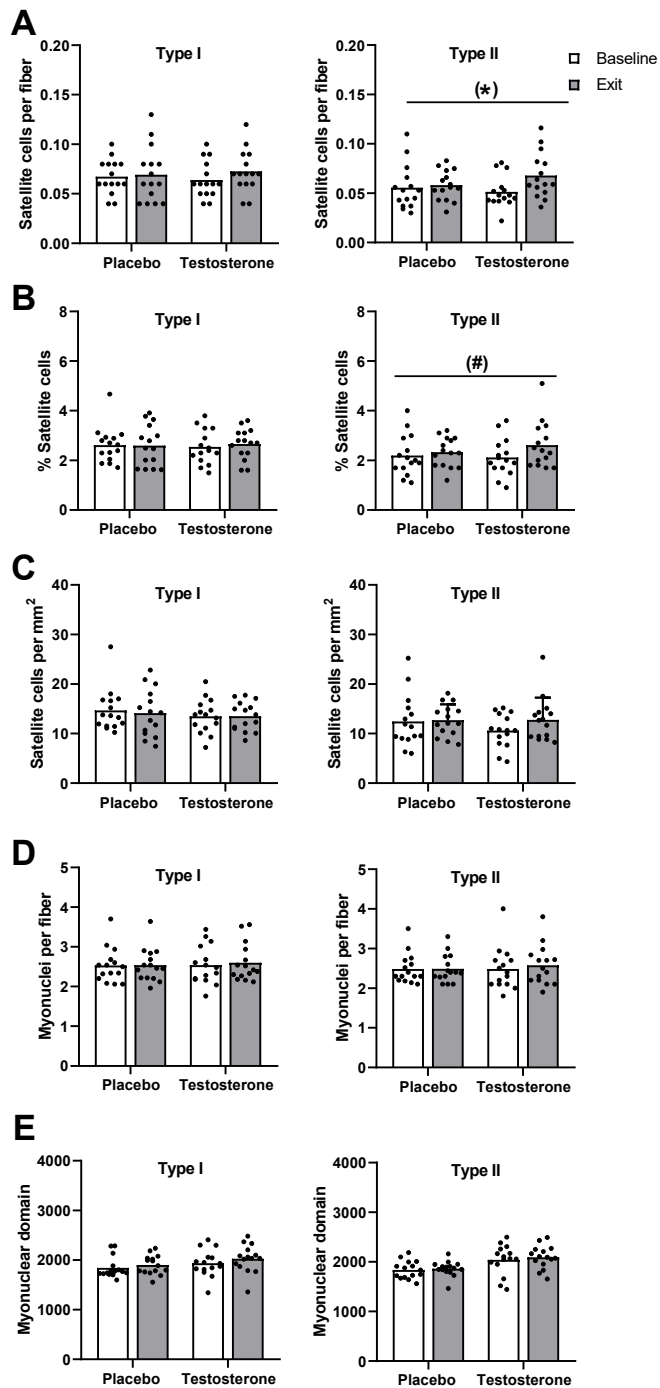


Figure 3.

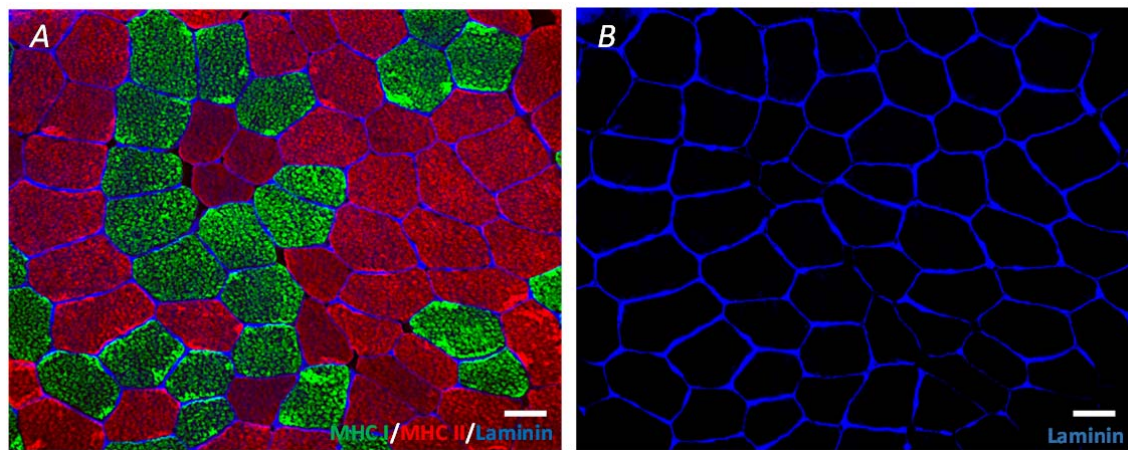


Figure 4.

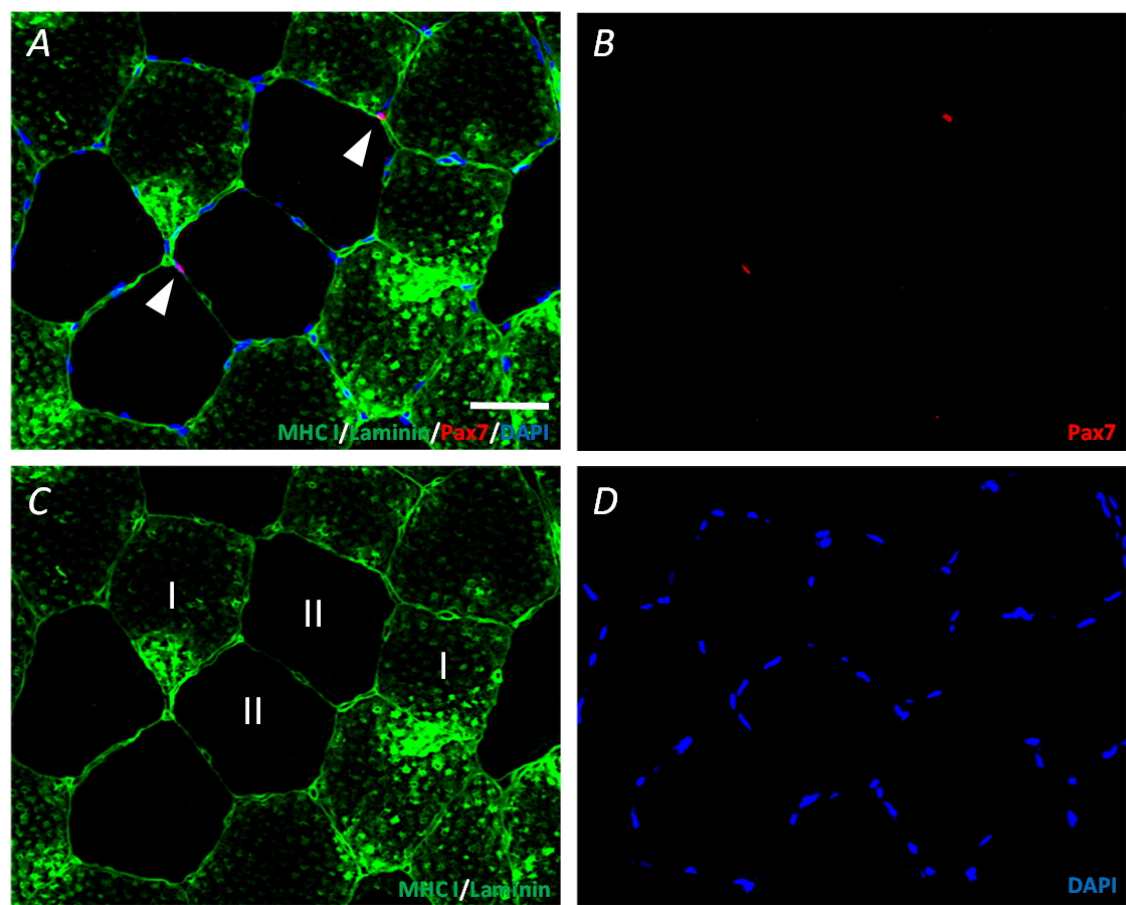


Figure 5.

