Distribution of myogenic progenitor cells and myonuclei is altered in women with vs. those without chronically painful trapezius muscle

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[1]*Institute of Sports Medicine, Department of Orthopaedic Surgery M, Bispebjerg Hospital, and Centre for Healthy Ageing, Faculty of Health Sciences, University of Copenhagen, and* [2]*National Research Centre for the Working Environment, Copenhagen; and* [3]*Institute of Sports Science and Clinical Biomechanics, Faculty of Health Sciences, University of Southern Denmark, Odense, Denmark*

Mackey AL, Andersen LL, Frandsen U, Suetta C, Sjøgaard G. Distribution of myogenic progenitor cells and myonuclei is altered in women with vs. those without chronically painful trapezius muscle. *J Appl Physiol* 109: 1920–1929, 2010. First published October 7, 2010; doi:10.1152/japplphysiol.00789.2010.—It is hypothesized that repeated recruitment of low-threshold motor units is an underlying cause of chronic pain in trapezius myalgia. This study investigated the distribution of satellite cells (SCs), myonuclei, and macrophages in muscle biopsies from the trapezius muscle of 42 women performing repetitive manual work, diagnosed with trapezius myalgia (MYA; 44 ± 8 yr; mean $\pm$ SD) and 20 matched healthy controls (CON; 45 ± 9 yr). Our hypothesis was that muscle of MYA, in particular type I fibers, would demonstrate higher numbers of SCs, myonuclei, and macrophages compared with CON. SCs were identified on muscle cross sections by combined immunohistochemical staining for Pax7, type I myosin, and laminin, allowing the number of SCs associated with type I and II fibers to be determined. We observed a pattern of SC distribution in MYA previously only reported for individuals above 70 yr of age. Compared with CON, MYA demonstrated 19% more SCs per fiber associated with type I fibers (MYA 0.098 ± 0.039 vs. CON 0.079 ± 0.031; $P < 0.05$) and 40% fewer SCs associated with type II fibers (MYA 0.047 ± 0.017 vs. CON 0.066 ± 0.035; $P < 0.05$). The finding of similar numbers of macrophages between the two groups was not in line with our hypothesis and suggests that the elevated SC content of MYA was not due to heightened inflammatory cell contents, but rather to provide new myonuclei. The findings of greater numbers of SCs in type I fibers of muscle subjected to repeated low-intensity work support our hypothesis and provide new insight into stimuli capable of regulating SC content.

Pax7; immunohistochemistry; low-threshold motor units; human skeletal muscle; macrophages

CHRONIC TENDERNESS AND TIGHTNESS of the upper trapezius muscle, clinically termed trapezius myalgia, is frequent among women performing repetitive tasks, such as computer work (18). At a morphological level, findings in biopsies from myalgic muscle indicate the presence of large type I fibers, megafibers, poor capillarization, and evidence of disturbed mitochondrial organization (5, 17, 23, 24), reviewed elsewhere (16, 46). Supporting the unusual findings regarding type I fibers in particular is the "Cinderella hypothesis," put forward as an explanation for myalgia almost 20 yr ago (16). This is based on the premise that low-threshold motor units are the first to be recruited, are continuously active during a sustained activation of the muscle, and are thus at risk of being damaged (16, 46). However, whether the extent of this overloading results in heightened inflammatory and myogenic activity has not been investigated previously.

Ever since their discovery at the periphery of a frog muscle fiber almost 50 yr ago (31), there has been growing interest in satellite cells (SC) as a self-renewing reservoir of myogenic potential for postnatal regeneration, growth, and maintenance of skeletal muscle. There is focus not only on the behavior of SCs in healthy individuals (7, 15, 20, 22, 27, 30, 32, 44, 45), but in muscle diseases of many kinds (33, 39, 42, 48). For example, heightened SC activity in patients suffering from muscle dystrophy has been reported (42, 48). There is also evidence to suggest that the continuous cycles of degeneration and regeneration associated with some muscle diseases eventually exhaust the reservoir of repair cells, leaving the muscle with a poorer regenerative capacity (12, 39, 42). In healthy individuals, the majority of studies investigating SCs have reported expansion of the SC pool with heavy resistance training (20, 22, 27, 36, 45), where the role of the new SCs in donating new myonuclei under conditions of hypertrophy and repair seems clear. However, evidence is also emerging for enhanced SC content with lower muscle loads (8, 28, 30, 45), where injury and hypertrophy are not as evident, supporting a role for SCs over a broader physiological range. There are, therefore, good grounds for the hypothesis that SC and myonuclear content are altered in myalgic muscle exposed to prolonged low-level activation. Such information would not only provide valuable insight into the pathophysiological spectrum of SC activity, but, from a clinical perspective, may contribute toward a better understanding of the myogenic stress experienced by painful muscles.

The aim of this study, therefore, was to compare the content of SCs and myonuclei in biopsies from myalgic muscle with those from matched healthy controls. In addition, we examined the content of macrophages, which are known to have both pro- and anti-inflammatory roles in removing damaged material and promoting muscle repair by various means, including stimulating proliferation of myoblasts (9, 40, 43). In further support of an association between inflammation and myalgia, there is recent direct evidence for increased levels of the proinflammatory cytokine tumor necrosis factor-α in the skeletal muscle of rats performing low-force, high-repetition tasks for 12 wk (14), suggesting that chronic repetitive office work could initiate an inflammatory response in the working muscles. We hypothesized that, compared with controls, myalgic muscle would *1*) contain higher numbers of macrophages, and

Table 1. *Physical activity*

	Moderate	Vigorous
CON	$3,156 \pm 2,036$	$1,824 \pm 1,956$
MYA	$2,749 \pm 2,395$	$670 \pm 1,586$[a]

Values are means $\pm$ SD in metabolic equivalent (MET)·min·wk^{-1}. Documentation is shown of the amount of moderate and vigorous physical activity performed per week. MYA, trapezius myalgia group; CON, healthy control group. [a]MYA < CON ($P < 0.05$).

2) be characterized by greater numbers of SCs and myonuclei, particularly in type I fibers.

MATERIALS AND METHODS

Subjects

A case-control study was performed during the period from September 2005 to March 2006 in Copenhagen, Denmark. The study design and procedure of recruitment have been described in detail previously (41). Briefly, 42 female office workers with myalgia in their trapezius muscle (MYA; 44 ± 8 yr of age, 165 ± 6 cm, 72 ± 15 kg, means $\pm$ SD) and 20 control women comparable with regard to job-type, age, weight, and height, but without neck myalgia, (CON; 45 ± 9 yr of age, 167 ± 6 cm, 70 ± 11 kg, means $\pm$ SD) participated. The participants in both CON and MYA were active in the labor market and recruited from workplaces with monotonous and repetitive work tasks, mostly office and computer work. The participants in each group had to meet a number of self-reported and clinically diagnosed criteria, which have been described in detail recently (18). Participants with serious clinical conditions, such as previous trauma, life-threatening diseases, whiplash injury, cardiovascular diseases, or arthritis in the neck and shoulder were excluded from both groups. All women in MYA were clinically diagnosed with trapezius myalgia, where the main criteria for a positive diagnosis were *1*) chronic or frequent pain in the neck area, *2*) tightness of the upper trapezius muscle, and *3*) palpable tenderness of the upper trapezius muscle (18, 41). All participants were informed of the purpose and content of the project and gave written, informed consent to participate in the study, which conformed to The Declaration of Helsinki and was approved by the local ethics committee (KF 01–138/04). Muscle mechanical function and muscle fiber-type composition have recently been reported for the same group of women with trapezius myalgia compared with matched controls (1, 3, 5).

Work Exposure

The extent of work exposure was assessed by questionnaire. Participants were asked to answer three questions relating to the amount of work time spent *1*) in front of a computer, *2*) using the keyboard, and *3*) using the mouse. The answers they could choose from for each question were as follows: *1*) All of the time, *2*) ¾ of the time, *3*) ½ of the time, *4*) ¼ of the time, *5*) Rarely, or *6*) Never. The frequencies were calculated from these questionnaires. Analysis of the answers to the three questions regarding exposure to computer work revealed a tendency for MYA to spend more time using the keyboard than CON ($P = 0.078$). A similar proportion of time spent working at a computer or actively using the mouse was reported for MYA and CON.

Physical Activity

The participants completed an internet-based questionnaire about physical activity [a Danish version of the International Physical Activity Questionnaire Long Form Questionnaire (10)]. Total moderate and vigorous-intensity physical activity performed by the participants at work, transportation, housework/gardening, and leisure were converted to metabolic equivalent per minute per week, according to the guidelines for data processing of the International Physical Activity Questionnaire (17a). Documentation of the amount of physical activity is presented in Table 1 and shows that, while CON and MYA performed similar amounts of moderate physical activity, MYA performed a significantly lower amount of vigorous physical activity compared with CON ($P < 0.05$).

Pain Drawings

Study participants were presented with a body map with five defined regions: head, neck, upper back, right shoulder, and left shoulder (Fig. 1). They were asked to mark by pencil for each area whether pain was present (13). The analysis of the drawings was blinded regarding case or control. The percentage of individuals reporting pain for MYA and CON was calculated. As presented in Fig. 1, 98% of MYA subjects reported pain in the neck region, compared with 37% in CON. The proportion of MYA individuals reporting pain in the upper back, and left, and right shoulders ranged from 32 to 46%, compared with 0–11% in CON.
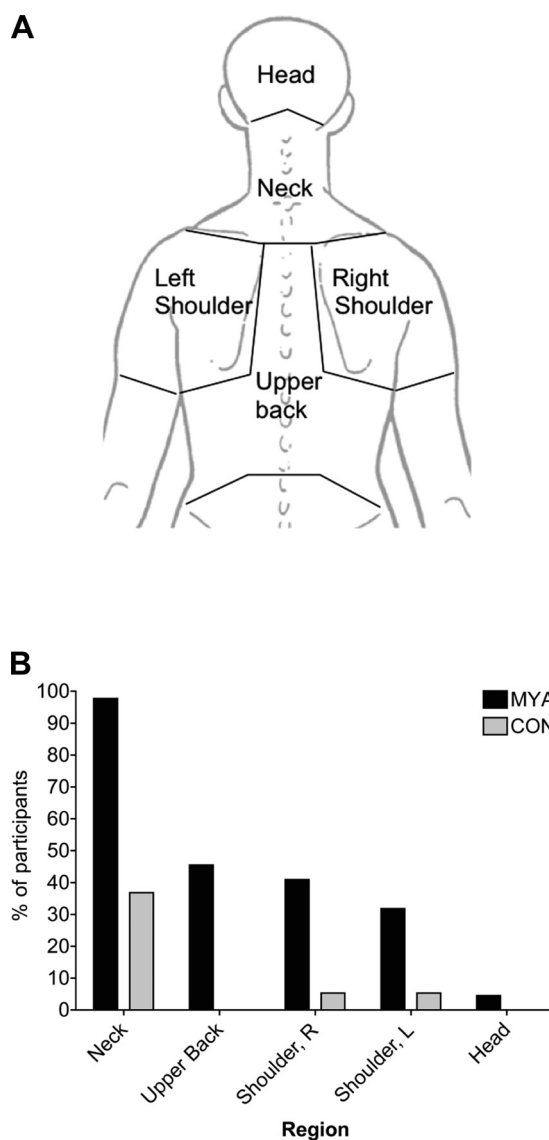

Fig. 1. *A*: body map with five regions defined (head, neck, upper back, right shoulder, and left shoulder) for pain drawings. *B*: overview of reports of upper body regional pain in trapezius myalgia (MYA) and healthy control (CON) groups. Data are presented as percentage of participants reporting pain for each region.

Muscle Biopsies

Muscle biopsies were extracted with a Bergstrom biopsy needle from the upper trapezius muscle at the midpoint between the 7th cervical vertebra and the acromion. Prior inspection with ultrasonography was carried out every time to determine the exact biopsy site. On extraction of the specimen, the fibers were aligned, embedded in Tissue-Tek, and frozen by immersion in isopentane, precooled to approximately $-160°C$ by liquid nitrogen. Samples were stored at $-80°C$. All biopsy samples were assigned a unique identification number, thus blinding the investigator to the participant's identity. Serial transverse sections (10 μm) were cut at $-24°C$ using a cryostat and picked up onto SuperFrost Plus glass slides (Menzel-Gläser, Braunschweig, Germany). The same person (A. L. Mackey) carried out all subsequent analysis. Biopsies were analyzed in batches, arranged by an investigator not involved in the analysis, such that each batch contained biopsies from both groups.

SCs

SC analysis was carried out by microscopic evaluation of cryosections immunohistochemically stained for Pax7, as previously used by others (7, 11, 25, 29, 32, 38, 44). A combination of immunoenzymatic and immunofluorescence methods was developed for this study to allow staining of Pax7, type I myosin, and laminin on the same section. All sections were fixed for 5 min with a 5% formaldehyde solution (Histofix, Histolab, Gothenburg, Sweden). Blocking of nonspecific binding sites was performed by incubation for 1 h with a freshly prepared blocking buffer [0.05 M Tris-buffered saline (TBS) containing 0.01% Triton, 1% bovine serum albumin (BSA), 1% skimmed milk powder, and 0.1% sodium azide]. SCs were labeled with a mouse anti-Pax7 antibody (cat. no. MO15020; Neuromics, Edina, MN), diluted 1:100 in the blocking buffer, and incubated overnight at 4°C. The next day, the slides were washed for 2×10 min in 0.05 M TBS containing 0.01% Triton. A biotinylated goat anti-mouse secondary antibody (cat. no. E0433; Dako Denmark, Glostrup, Denmark) was then applied, followed by Vector Elite ABC horseradish peroxidase kit (cat. no. PK6100; Vector Laboratories, Peterborough, UK). Horseradish peroxidase activity was visualized with the Immpact diaminobenzidine substrate (cat. no. SK-4105; Vector Lab-

oratories). The sections were then incubated for 2 h at room temperature with a mixture of 1:100 dilutions (in 0.05 M TBS containing 1% BSA) of the two primary antibodies mouse anti-A4.951 (cat. no. A4.951; Developmental Studies Hybridoma Bank, Iowa, IA) and rabbit anti-laminin (cat. no. Z0098; Dako), for visualization of type I myosin and laminin, respectively. Alexa Fluor 488 goat anti-rabbit (Molecular Probes cat. no. A11034; Invitrogen, Taastrup, Denmark) and Alexa Fluor 568 goat anti-mouse secondary antibodies (Molecular Probes, cat. no. A11031) were mixed together at concentrations of 1:200 in 0.05 M TBS, containing 1% BSA, and applied to the sections for 45 min. Washing in two changes of 0.05 M TBS was carried out between all steps. 4′,6-Diamidino-2-phenylindole (DAPI) in the mounting medium (Molecular Probes ProLong Gold anti-fade reagent, cat. no. P36935) stained the nuclei, rendering type I myosin red, laminin green, and nuclei blue. Sites of Pax7 antigenicity were stained brown, visible by light microscopy. See Fig. 2 for an example of this staining on one of the biopsies from this study.

SC Analysis

The aim of this analysis was to determine the number of Pax7+ cells and to distinguish whether they were associated with type I or type II fibers. To do this, a gray-scale image of the fluorescent laminin staining was captured (with an Olympus DP71 digital camera, mounted on an Olympus BX51 microscope using a $\times 4$ objective with a $\times 0.5$ camera mount), inverted, and printed out on an A4 page. Viewing the section with $\times 20$ and $\times 40$ objectives, Pax7 cells were identified, and their locations recorded on the respective fibers on the laminin print. A gray-scale image of the fluorescent type I myosin staining, captured and inverted in the same manner as the laminin image, was printed on a transparent A4 sheet. Overlaying this on the laminin print revealed whether the Pax7 cells were associated with type I (stained) or type II (unstained) fibers. The number of Pax7 cells associated with type I or type II fibers was counted separately and expressed relative to the total number of type I or type II fibers included in the assessment. The number of Pax7 cells was also expressed relative to fiber area and as a proportion of the number of myonuclei.
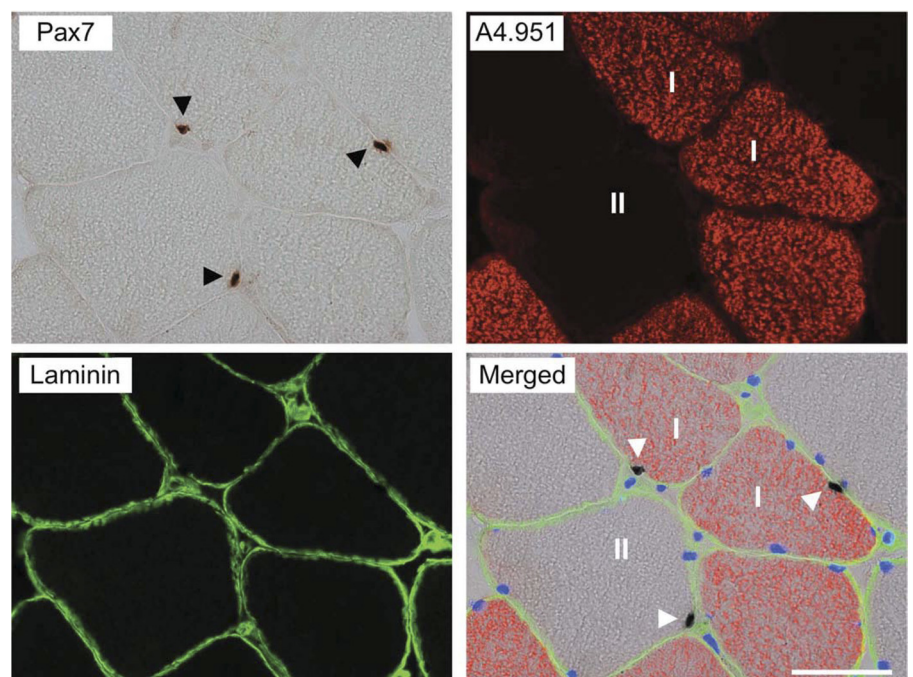

Fig. 2. Adapted immunohistochemical detection of Pax7 cells, type I myosin, and laminin on a single cross section of healthy trapezius muscle (CON group). In this series of images, three Pax7 cells (brown) are visible, two of which are associated with type I fibers (red), and one with a type II fiber (unstained). Laminin staining (green) defines the fiber borders. Note the immediate proximity to a laminin-rich capillary of two of the Pax7 cells in this image. Scale bar = 50 μm.

Myonuclei

All measurements relating to myonuclei were made from the sections stained for the assessment of SCs. On these sections, the staining for nuclei, laminin, and type I myosin was visible by fluorescence microscopy (Fig. 2). The number of fibers with central nuclei was determined by viewing each section with a ×20 objective and recording the number of type I fibers containing one or more central myonuclei. The same assessment was then made for type II fibers. A nucleus was considered centrally located if a gap between the nucleus and the laminin-rich basement membrane was visible at this magnification. For measurements relating to the number of myonuclei per fiber and the myonuclear domain, digital images were captured using a ×20 magnification (×0.5 camera mount) with a triple-band filter adjusted so that laminin and type I fiber staining were visible. Using the program Cell^F (Olympus Life and Material Science Europe, Hamburg, Germany), type I fibers (A4.951+) were identified on this image, and the number of fibers recorded. With the aid of the freehand drawing tool, the laminin-defined edges of these fibers were delineated manually, and the combined area of the fibers was generated. Myonuclei were identified individually through the microscope using a ×40 objective, and their location was marked on the image. This was carried out for all of the marked fibers, and the total number of myonuclei was recorded. This procedure was repeated for type II fibers (A4.951−). Data generated from this analysis were *1*) the mean fiber area, *2*) the number of myonuclei/fiber, and *3*) the area of cytoplasm per myonucleus (myonuclear domain). These data were generated separately for type I and type II fibers.

Myofiber Remodeling and Cell Activity

Myofiber regeneration was assessed by staining three serial sections for *1*) embryonic myosin, *2*) neonatal myosin, and *3*) CD56 as an additional indicator of regeneration and the innervation status of the fibers (49). After fixation for 5 min in a 5% formaldehyde solution (Histofix), sections were incubated overnight at 4°C with *1*) mouse anti-embryonic myosin (F1.652; Developmental Studies Hybridoma Bank), or *2*) mouse anti-neonatal myosin (NCL-MHCn; Novocastra, Newcastle upon Tyne, UK). Both primary antibodies were mixed with rabbit anti-laminin (cat. no. Z0098; Dako) to define the fiber borders. Alexa Fluor 488 goat anti-rabbit (Molecular Probes, cat. no. A11034) and Alexa Fluor 568 goat anti-mouse (Molecular Probes, cat. no. A11031) secondary antibodies were applied to the sections to visualize primary antibody binding. DAPI in the mounting medium (Molecular Probes ProLong Gold anti-fade reagent, cat. no. P36935) stained the nuclei, rendering embryonic myosin and neonatal myosin red, laminin green, and nuclei blue (see Fig. 3 for example). *3*) Staining for CD56 was performed in the same manner, with a mouse anti-CD56 antibody (cat. no. 347740; Becton Dickinson, San Jose, CA), but double-stained with Ki67 (cat. no. CP249; Biocare Medical, Concord, CA) instead of laminin, with the dual purpose of evaluating the presence of cells in the active phase of the cell cycle (Ki67+), as recently described (29), as well as the proportion of fibers demonstrating immunoreactivity for the CD56 antigen. The number of embryonic myosin, neonatal myosin, and CD56 positive fibers was counted from the respective stainings and expressed as a percentage of the total number of fibers on that section. For the assessment of actively dividing cells, Ki67+ cells were identified, and whether these cells were CD56 positive (Ki67+CD56+) or negative (Ki67+CD56−) was recorded. The values were expressed relative to the number of fibers in the cross section as cells per 100 fibers. The total number of Ki67+ cells per 100 fibers {[(Ki67+CD56+) + (Ki67+CD56−)] × 100} was also calculated from these data.

Macrophages

Macrophages were identified on cryosections by using an indirect double immunofluorescence labeling protocol, as follows. The sec-

tions were incubated simultaneously overnight with a primary antibody against macrophages (mouse anti-CD68, cat. no. M0718; Dako) and the basement membrane component laminin (cat. no. Z0098; Dako), both diluted in 0.05 M TBS (containing 1% BSA) to a concentration of 1:100. Visualization of primary antibody binding was achieved by adding a mixture of the two secondary antibodies Alexa Fluor 568 goat anti-mouse (Molecular Probes, cat. no. A11031) and Alexa Fluor 488 goat anti-rabbit (Molecular Probes, cat. no. A11034). DAPI in the mounting medium (Molecular Probes ProLong Gold antifade reagent, cat. no. P36931) stained the nuclei, which rendered macrophages red, laminin green, and nuclei blue (Fig. 4). The relative number of macrophages was quantified by counting the number of CD68+ cells on the cross section and then expressing this number relative to the total number of muscle fibers (×100).

Statistics

The investigator performing the biopsy analyses was blinded. Subsequently, the data were unblinded and statistically analyzed by an investigator not involved in the muscle biopsy analyses. Differences in the main variables between MYA and CON were tested with two-way ANOVA. Factors included in the model were group (MYA, CON), fiber type (types I and II), and group by fiber type. Statistical significance was accepted at the 0.05 level. Test of one-sided hypotheses was deemed significant if a two-sided P value was <0.10. Data are presented as means ± SD. Regression analysis was performed on the fiber area and myonuclear number data producing Pearson's correlation coefficient for each analysis.

RESULTS

SCs

Thirty-seven biopsies from MYA and 17 biopsies from CON were considered to be of adequate quality and size to be included in the analysis. A mean of 627 ± 264 (SD) fibers (418 ± 167 type I fibers, 209 ± 142 type II fibers) per biopsy was evaluated for MYA, and a mean of 683 ± 199 (SD) fibers (439 ± 149 type I fibers, 244 ± 90 type II fibers) per biopsy was evaluated in the CON group. SCs are presented as Pax7 cells per fiber number, per myofiber area, and as a proportion of the number of myonuclei (Table 2).

Pax7/fiber. A significant group-by-fiber interaction was observed for the number of Pax7 cells per fiber ($P = 0.0005$), with a significant main effect of fiber type ($P < 0.0001$). Post hoc analyses revealed a 19% higher concentration of Pax7 cells associated with type I fibers in MYA compared with CON ($P = 0.0226$). Conversely, the number of Pax7 cells associated with type II fibers was 40% lower in MYA compared with CON ($P = 0.0359$).

Pax7/area. When corrected for fiber area, a significant group-by-fiber interaction was observed for the number of Pax7 cells ($P = 0.0082$), with a significant main effect of fiber type ($P = 0.0340$). Again, greater numbers of Pax7 cells per type I fiber were observed in MYA compared with CON ($P = 0.0281$).

Pax7/myonuclei. A significant group-by-fiber interaction was observed for the number of Pax7 cells expressed as a proportion of myonuclei ($P = 0.0136$), with a significant main effect of fiber type ($P < 0.0001$). The number of Pax7 cells per type I fiber was elevated in MYA compared with CON (one-tailed, $P = 0.0488$).

In addition, the number of Pax7 cells associated with type I fibers was significantly greater than the number observed per type II fiber for MYA ($P < 0.0001$), but not for CON, for Pax7
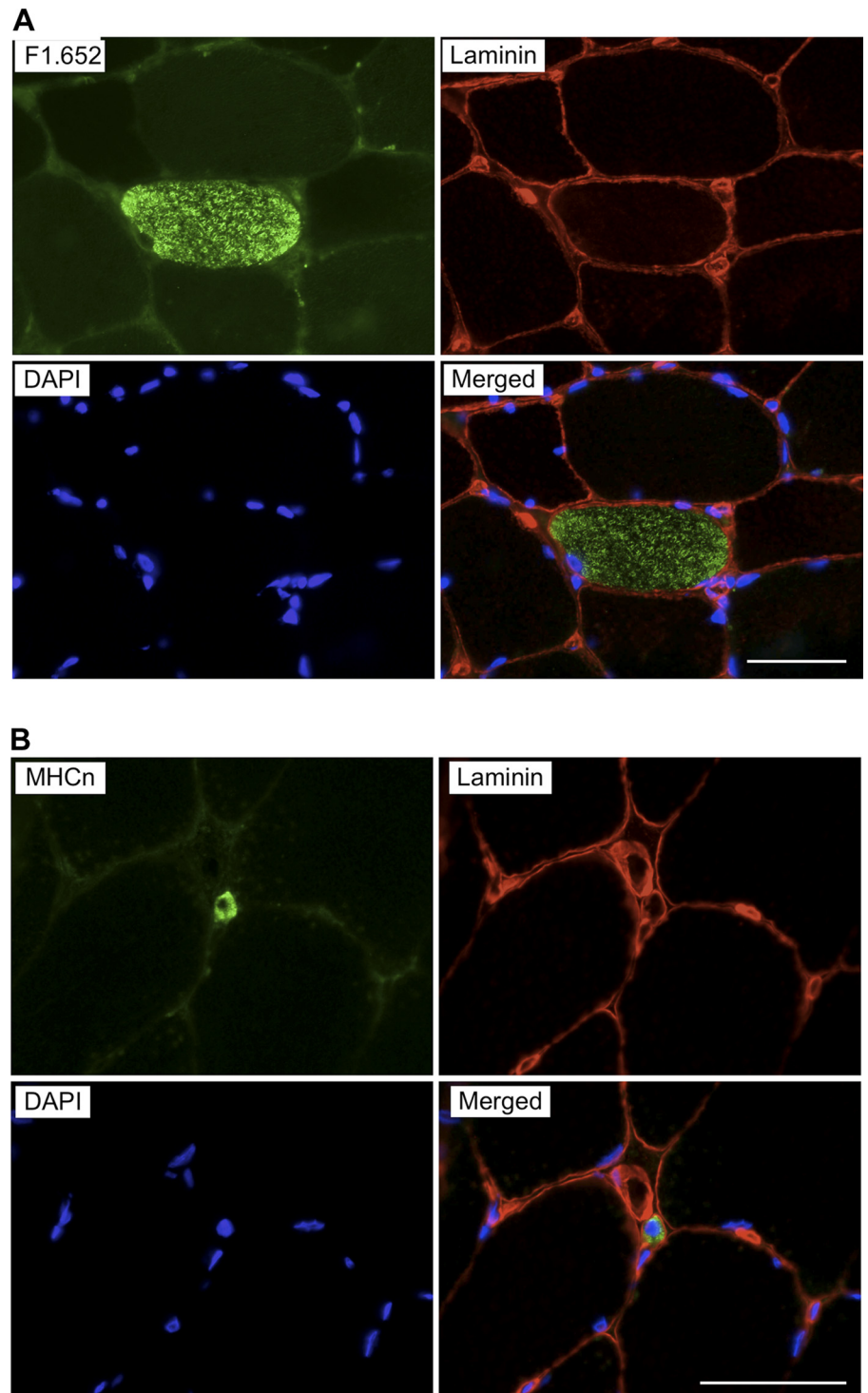
A

B

Fig. 3. Immunohistochemical detection of embryonic myosin (F1.652, green; *A*) or neonatal myosin (MHCn, green; *B*), together with laminin (red) and nuclei (blue) on a cross section of trapezius muscle from the MYA group. These fibers were rare in the muscle samples of this study and indicate ongoing regenerative processes at the time of biopsy sampling. DAPI, 4′,6-diamidino-2-phenylindole. Scale bars = 50 μm.

cells per fiber number, per myofiber area, and as a proportion of the number of myonuclei.

Myonuclei

A significant group-by-fiber interaction ($P = 0.0290$) and a tendency for a main effect of fiber type ($P = 0.0793$) were observed for the number of fibers with centrally located myonuclei (Table 2). Post hoc analyses revealed a significantly lower proportion of type II fibers with central nuclei in MYA,

compared with MYA type I fibers ($P = 0.0005$) and CON type II fibers ($P = 0.0209$).

With regard to the number of myonuclei per fiber, a mean of 58 ± 7 fibers [31 ± 3 (SD) type I fibers, and 28 ± 4 type II fibers] per biopsy was evaluated. A significant group-by-fiber interaction was observed ($P = 0.0318$), with a significant main effect of fiber type ($P = 0.0006$). Post hoc testing uncovered a higher number of myonuclei associated with type I fibers vs. type II fibers in MYA ($P < 0.0001$) and a tendency for a higher
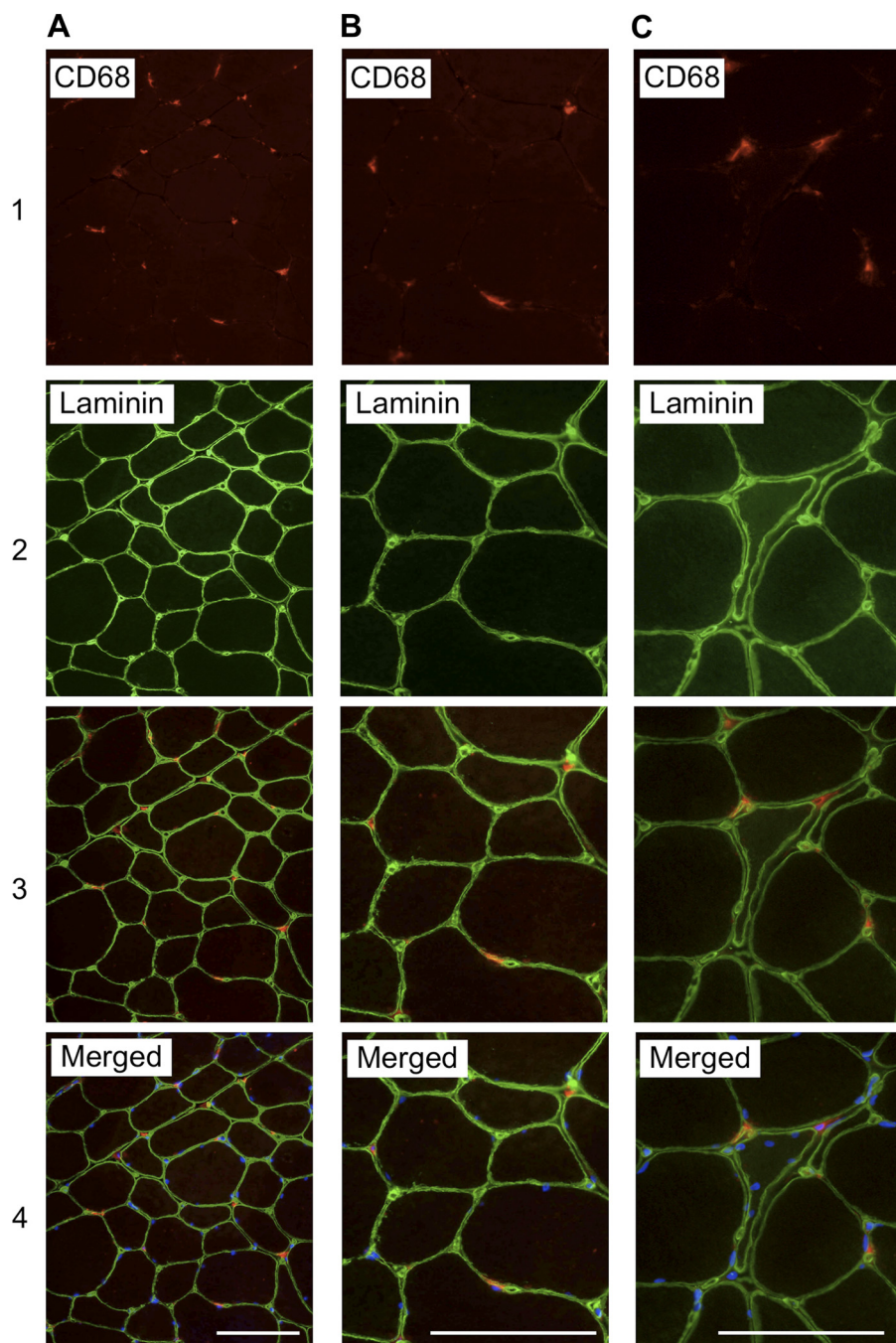
Fig. 4. Series of images of typical immunohisto-chemical stainings for macrophages (CD68+ cells) and laminin. *A*, *B*, and *C*: series of images of one section. *Row 1*: macrophage staining in red. *Row 2*: laminin in green. *Row 3*: combined image of *rows 1* and *2*. *Row 4*: combined image of *rows 1* and *2* and DAPI in blue. *B*: a higher magnification (×40) of the region shown in *A* (×20). *C*: an example of an unusually shaped fiber containing a centrally located nucleus, visible in the merged image *C4*. Scale bars = 100 μm.

number of myonuclei per type I fiber in MYA compared with CON ($P = 0.0513$, one-tailed). Myonuclear domain size demonstrated a significant main effect of fiber type ($P < 0.0001$), but not group or interaction, with type I fibers displaying a larger myonuclear domain than type II fibers (Table 2).

Macrophages

The average number of muscle fibers included in the analyses per biopsy sample was 733 ± 429 for CON and 667 ± 312 for MYA. At baseline, CON and MYA had 28 ± 11 and 26 ± 11 macrophages per 100 muscle fibers, respectively. These values were not significantly different from each other.

Myofiber Remodeling and Cell Activity

Fisher's exact test revealed a tendency for a higher prevalence of biopsies with one or more embryonic myosin positive fiber in MYA compared with CON ($P = 0.089$). The number of biopsies demonstrating embryonic myosin positive fibers was 0 (out of 19) for the CON group and 7 (out of 42) in the MYA group. No differences were observed between groups for the incidence of neonatal positive fibers (only observed in one biopsy) or CD56+ fibers. CD56+ fibers were observed in 78% of CON and 79% of MYA. CON and MYA also demonstrated similar numbers of biopsies containing one or more Ki67+CD56+ cells, Ki67+CD56− cells, and total number of

Table 2. *Muscle fiber and cellular characteristics*

	All Combined	Type I Fibers	Type II Fibers
Fiber area, μm^2			
CON	4,579 ± 928	5,010 ± 1,107	3,919 ± 1,129[b]
MYA	4,638 ± 937	5,193 ± 1,110	3,501 ± 977[b]
Fibers with central nuclei, %			
CON	3.3 ± 2.5	3.3 ± 2.9	3.6 ± 2.9
MYA	3.1 ± 2.1	3.7 ± 2.8	1.6 ± 2.8[a,b]
Myonuclei/fiber			
CON	2.2 ± 0.4	2.3 ± 0.4	2.1 ± 0.7
MYA	2.3 ± 0.5	2.6 ± 0.6[c]	2.0 ± 0.6[b]
Fiber area/myonucleus[d], μm^2			
CON	1,981 ± 256	2,115 ± 307	1,811 ± 189
MYA	1,859 ± 307	2,013 ± 362	1,659 ± 329
Pax7/fiber			
CON	0.073 ± 0.028	0.079 ± 0.031	0.066 ± 0.035
MYA	0.082 ± 0.026	0.098 ± 0.039[a]	0.047 ± 0.017[a,b]
Pax7/mm² fiber area			
CON	16.0 ± 4.3	15.9 ± 5.6	16.2 ± 5.6
MYA	16.8 ± 4.4	19.3 ± 5.7[a]	14.3 ± 5.7[b]
Pax7/myonuclear no., %			
CON	2.9 ± 0.7	3.1 ± 1.1	2.7 ± 1.1
MYA	3.3 ± 0.9	3.6 ± 1.1[c]	2.3 ± 1.1[b]

Values are means ± SD. Data are presented for combined fibers types and differentiated according to type I and type II fibers. The number of satellite cells (Pax7+) are given, expressed relative to fiber number, mm² of tissue, and myonuclear number. The number of fibers with central nuclei, myonuclei per fiber, and the area surrounding each myonucleus are also shown. [a]MYA vs. CON ($P < 0.05$). [b]Type II fibers vs. corresponding value for type I fibers ($P < 0.001$). [c]MYA vs. CON (one-tailed, $P \le 0.05$). [d]Main effect of fiber type only ($P < 0.0001$).

Ki67+ cells, expressed per 100 fibers. Ki67+CD56+ cells were observed in three of the MYA biopsies and none of CON.

Fiber Area

A tendency for a group-by-fiber interaction ($P = 0.0587$) and a significant main effect of fiber type ($P < 0.0001$) was observed for fiber area. Further analyses revealed smaller type II fibers compared with type I for both MYA and CON ($P < 0.0001$). A post hoc comparison between type II fiber area of MYA and CON gave a P value of 0.1215.

Regression analyses of fiber area and the number of myonuclei per fiber revealed a significant strong positive relationship (R: range from 0.53 to 0.91, $P < 0.05$) for both fiber types in MYA and CON (Fig. 5).

DISCUSSION

The central finding of this study is that type I myofibers in the trapezius muscle of women exposed to repetitive low-force tasks and suffering from myalgia have a higher density of SCs and myonuclei, compared with those of healthy matched controls. A recent study on these same individuals uncovered the presence of unusually hypertrophied type I muscle fibers, megafibers, in MYA (5). These fibers, on average larger than 10,000 μm^2, were present to a significantly higher extent in MYA compared with CON (5) and potentially belong to "Cinderella" motor units. The finding of increased SC content of type I fibers is thus in line with previous suggestions of a chronic loading of type I fibers in myalgia (16, 23). The MYA individuals in this study experienced more pain than CON in their upper back muscles and demonstrated a tendency to spend more time using the keyboard while performing computer work than CON. Our findings indicate that painful muscle fibers under prolonged work exposure have a need for greater numbers of SCs than muscle not exposed to this pattern of recruitment.

SCs and Myonuclei

Using an adapted form of SC immunohistochemistry, which facilitates the determination of the distribution of SCs with regard to type I and II fibers on a single cryosection, we observed a greater SC content of type I fibers in MYA than CON when expressed relative to fiber number and when corrected for fiber size. The fact that MYA SC number expressed relative to the number of myonuclei only demonstrated a tendency to differ from CON is likely due to the elevated numbers of myonuclei detected in type I fibers in MYA. According to the myonuclear domain theory, each myonucleus can support an increasing volume of cytoplasm until a limit is reached, after which new myonuclei are required, if the fiber is to expand further. While we observed a stronger positive correlation between fiber area and myonuclear number for type II fibers than type I in both MYA and CON, exactly how tightly coupled these variables are is unclear (6, 22, 36). In addition to evidence of a poor correlation in mature murine muscle, it has also been suggested that myonuclear capacity can become impaired with aging (6). In the context of myalgic muscle, the hypothesis of a greater load on type I fibers potentially represents two scenarios for myonuclei: *1*) a higher density of myonuclei is required to sustain a higher rate of protein turnover, a consequence of the nature of the chronic loading, or *2*) the higher density of myonuclei reflects an impaired capacity of the existing myonuclei, creating a demand for additional myonuclei. In either situation, it is plausible that the role of the elevated SC content in the type I fibers of MYA in this study is to meet the demand for extra myonuclei. Furthermore, the negligible number of actively dividing SCs (Ki67+ CD56+) at the time of biopsy sampling suggests that this is a chronic, rather than an acute (29), myogenic response.

In contrast to the greater SC content of type I fibers in MYA, we observed fewer SCs associated with type II fibers in MYA compared with CON. When corrected for fiber area or myonuclear number though, this difference was no longer present, suggesting that our finding of differing distributions in type II fibers is due to a smaller size or myonuclear density of type II fibers in MYA. The similar myonuclear content of type II fibers in MYA and CON does not support the latter, while the outcome of the statistical analysis on the fiber area data indicates that we cannot exclude the possibility that MYA have smaller type II fibers than CON. While we did not have a measure of the amount of vigorous physical activity performed specifically by the trapezius muscle in our subjects, the lower amount of general vigorous physical activity reported by MYA compared with CON is interesting in relation to type II fibers since, in contrast to low-threshold type I motor units, the higher threshold type II motor units would only be recruited during vigorous physical activity. It can be further speculated that the significantly lower proportion of type II fibers with central nuclei in MYA, compared with type II fibers for CON and MYA type I, tentatively supports the lower exposure to vigorous physical activity. It could be expected that type II fibers subjected to
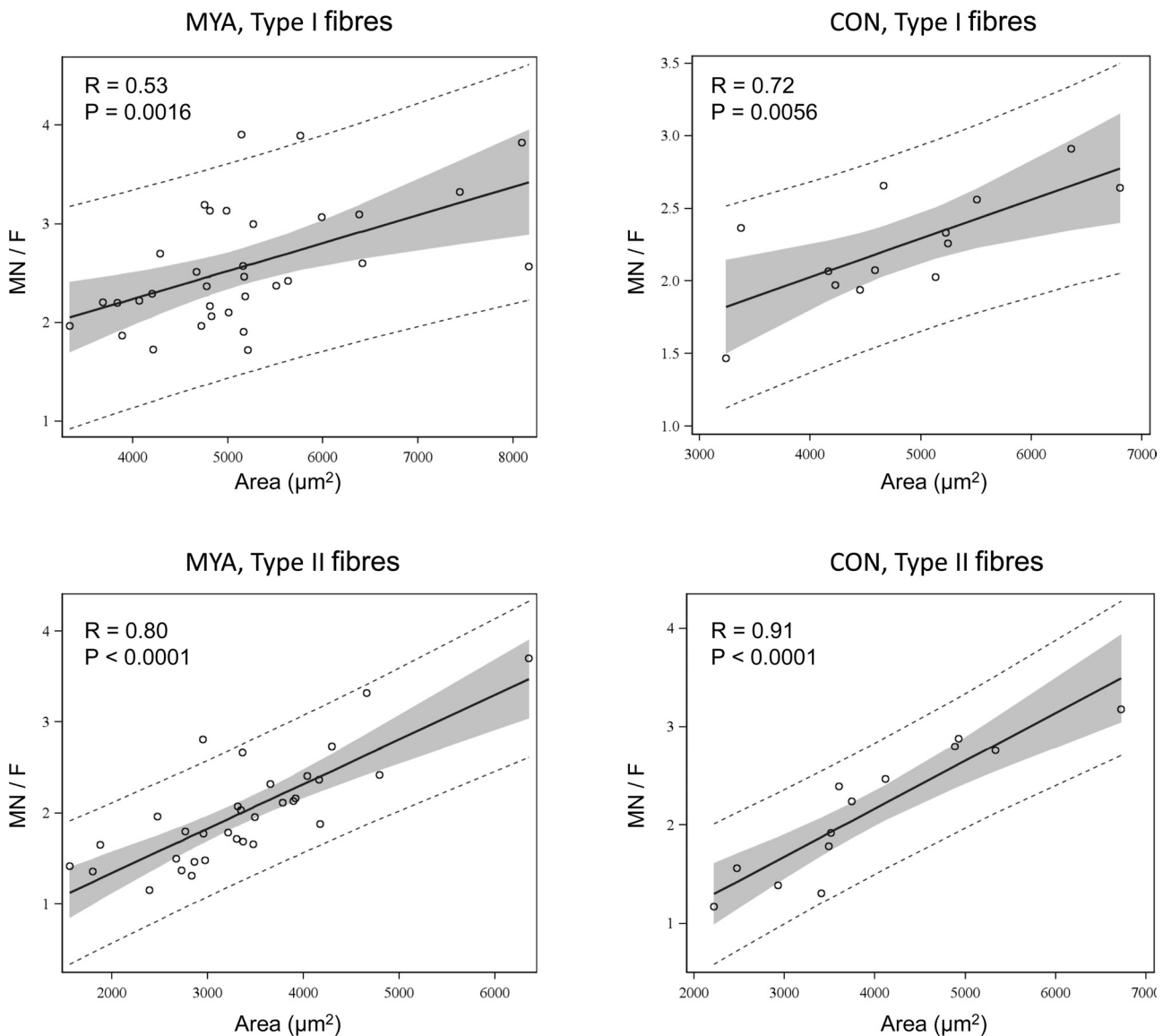
Fig. 5. Graphical illustration of the relationship between fiber area and the number of myonuclei per fiber (MN/F) for type I and II fibers in biopsies from the trapezius muscle of MYA and CON. 95% confidence limits and 95% prediction limits are denoted by the shaded area and dashed lines, respectively.

hard physical work would experience more regular cycles of repair, which would result in the presence of central nuclei in these fibers. However, this hypothesis requires substantiation, as the data on vigorous physical activity are self-reported at a whole body level and not specific for the trapezius muscle. We are, therefore, currently investigating this in a model in which the trapezius muscles are specifically targeted with heavy loading. The lack of difference in the percentage of type I fibers with central nuclei between MYA and CON is perhaps not surprising, given that the overloading of type I fibers in myalgia is at a low level, unlikely to induce muscle damage. These data are in contrast to a previous report of an increased prevalence of fibers with central nuclei in male myalgia patients, compared with controls (21). The reason for this discrepancy is unclear, and it is difficult to compare the two studies directly, given that the presence of central nuclei provides an indication of cycles of damage and repair that have occurred over years, not to mention the different sexes and

nature of repetitive work performed in the two studies. In agreement with the study by Kadi et al. (21), though, we observed a tendency for MYA to have more embryonic myosin-positive fibers than CON, indicating active muscle remodeling in MYA. In addition to the role of SCs in muscle growth, repair of skeletal muscle is a well-recognized function of SCs. Our findings of a greater number of embryonic myosin-positive fibers in MYA support this function and, together with the finding of a higher density of myonuclei in type I fibers, provide a likely explanation for the elevated SC number observed in MYA type I fibers.

Type I vs. II Fibers

In addition to the differences between the two groups, SC number was significantly higher in type I fibers compared with type II fibers for MYA, but not CON, when expressed relative to fiber number, fiber area, or myonuclear number. It is only

recently that reports of different numbers of SCs associated with type I or type II fibers have emerged. It appears that the muscle under investigation and the age of the individuals play a role in this heterogeneity, since biopsies from the vastus lateralis of young healthy individuals demonstrate similar numbers of SCs in type I and II fibers (19, 44). In contrast, type II fibers in vastus lateralis, but not deltoid, muscle (45) of healthy old individuals have been reported to contain fewer SCs compared with type I fibers (44, 45). Our finding of higher numbers of SCs in type I fibers compared with type II fibers in MYA mirrors this pattern observed in older individuals, despite the participants in our study having a mean age of 44 yr, compared with the mean ages of 76 and 73 yr in other reports of altered distribution of SCs with fiber type (44, 45), suggesting that inactivity, rather than age per se, plays a more significant role in the distribution of SCs according to muscle fiber type. While previous studies on resistance training of myalgic muscles do not provide any indication of hampered hypertrophy, and indeed have been shown to ameliorate chronic muscle pain (2, 35), whether the SC pool of this patient group can respond in a similar manner to healthy muscle remains to be established. Furthermore, in the context of age-associated loss of muscle mass and strength, it would be valuable to know if the already "aged" distribution of SCs in myalgic muscle would contribute to a predisposition to, or an accelerated development of, sarcopenia in this population.

Inflammatory Cells

Infiltration of inflammatory cells of various types is a feature of pathological, injured, and aging muscle (26, 34, 37, 47). A role for macrophages in the proliferation and growth of myoblasts in vitro is recognized (9), which was the basis for our hypothesis that MYA muscle would demonstrate higher numbers of macrophages than CON. Our finding of similar numbers of macrophages and actively dividing cells (Ki67+) in MYA and CON was contrary to our hypothesis and suggests that heightened inflammatory cell activity is not a feature of myalgic muscle. Furthermore, in light of the potent stimulus, macrophages appear to provide for myoblasts (9), the results of the present study suggest that the greater SC content observed in MYA is not driven by greater numbers of macrophages, but is instead more likely to be a response to the demand for extra myonuclei, as discussed above. It should be noted, however, that we only examined a broad group of macrophages exhibiting immunoreactivity for the CD68 antigen, and we cannot rule out that investigation of subpopulations of macrophages or other inflammatory cell types would have resulted in a different outcome. A further limitation is that types and levels of cytokines released by the macrophages present in the biopsies were not evaluated. Despite these limitations, however, it is clear that our CD68 analysis does not support our hypothesis that myalgic muscle contains greater numbers of macrophages.

Conclusions

In conclusion, our findings of increased SC and myonuclear content in type I fibers of myalgic muscle compared with healthy controls are in line with our hypothesis and provide strong evidence for heightened myogenic activity, likely arising out of chronic exposure to low-threshold motor unit activation. This contributes to the growing body of evidence for enhancement of the SC pool under conditions in which hypertrophy and damage are not evident (8, 28, 30, 45), adding support to the idea of a role for SCs over a broader physiological range than conventionally accepted. Further work is required to determine whether such modest enhancements of the SC pool (19% in the present study) have any physiological importance for the muscle, either in the present stage, or as these individuals age. An additional feature of the myalgic muscle investigated in this study was a pattern of distribution of SCs, according to fiber type previously only observed in elderly individuals. The consequences of this for the muscle's capacity to respond to vigorous physical activity and to resist sarcopenia remain to be established.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

1. **Andersen LL, Holtermann A, Jorgensen MB, Sjogaard G.** Rapid muscle activation and force capacity in conditions of chronic musculoskeletal pain. *Clin Biomech (Bristol, Avon)* 23: 1237–1242, 2008.
2. **Andersen LL, Kjaer M, Sogaard K, Hansen L, Kryger AI, Sjogaard G.** Effect of two contrasting types of physical exercise on chronic neck muscle pain. *Arthritis Rheum* 59: 84–91, 2008.
3. **Andersen LL, Nielsen PK, Sogaard K, Andersen CH, Skotte J, Sjogaard G.** Torque-EMG-velocity relationship in female workers with chronic neck muscle pain. *J Biomech* 41: 2029–2035, 2008.
5. **Andersen LL, Suetta C, Andersen JL, Kjaer M, Sjogaard G.** Increased proportion of megafibers in chronically painful muscles. *Pain* 139: 588–593, 2008.
6. **Bruusgaard JC, Liestol K, Gundersen K.** Distribution of myonuclei and microtubules in live muscle fibers of young, middle-aged, and old mice. *J Appl Physiol* 100: 2024–2030, 2006.
7. **Carlson ME, Suetta C, Conboy MJ, Aagaard P, Mackey A, Kjaer M, Conboy I.** Molecular aging and rejuvenation of human muscle stem cells. *EMBO Mol Med* 1: 381–391, 2009.
8. **Charifi N, Kadi F, Feasson L, Denis C.** Effects of endurance training on satellite cell frequency in skeletal muscle of old men. *Muscle Nerve* 28: 87–92, 2003.
9. **Chazaud B, Brigitte M, Yacoub-Youssef H, Arnold L, Gherardi R, Sonnet C, Lafuste P, Chretien F.** Dual and beneficial roles of macrophages during skeletal muscle regeneration. *Exerc Sport Sci Rev* 37: 18–22, 2009.
10. **Craig CL, Marshall AL, Sjostrom M, Bauman AE, Booth ML, Ainsworth BE, Pratt M, Ekelund U, Yngve A, Sallis JF, Oja P.** International physical activity questionnaire: 12-country reliability and validity. *Med Sci Sports Exerc* 35: 1381–1395, 2003.
11. **Crameri RM, Aagaard P, Qvortrup K, Langberg H, Olesen J, Kjaer M.** Myofibre damage in human skeletal muscle: effects of electrical stimulation vs voluntary contraction. *J Physiol* 583: 365–380, 2007.
12. **Decary S, Hamida CB, Mouly V, Barbet JP, Hentati F, Butler-Browne GS.** Shorter telomeres in dystrophic muscle consistent with extensive regeneration in young children. *Neuromuscul Disord* 10: 113, 2000.
13. **Ektor-Andersen J, Isacsson SO, Lindgren A, Orbaek P.** The experience of pain from the shoulder-neck area related to the total body pain,

self-experienced health and mental distress. The Malmo Shoulder-Neck Study group. *Pain* 82: 289–295, 1999.

14. **Elliott MB, Barr AE, Clark BD, Wade CK, Barbe MF.** Performance of a repetitive task by aged rats leads to median neuropathy and spinal cord inflammation with associated sensorimotor declines. *Neuroscience* 170: 929–941, 2010.

15. **Grounds MD, White JD, Rosenthal N, Bogoyevitch MA.** The role of stem cells in skeletal and cardiac muscle repair. *J Histochem Cytochem* 50: 589–610, 2002.

16. **Hägg GM.** Static work loads and occupational myalgia–a new explanation model. In: *Electromyographically Kinesiology*, edited by Andersen PA, Hobart DJ, and Danoff JV. Amsterdam: Elsevier Science (Biomedical Division), 1991, p. 141–144.

17. **Henriksson KG, Bengtsson A, Larsson J, Lindstrom F, Thornell LE.** Muscle biopsy findings of possible diagnostic importance in primary fibromyalgia (fibrositis, myofascial syndrome). *Lancet* 2: 1395, 1982.

17a.**IPAQ.** *Guidelines for Data Processing and Analysis of the International Physical Activity Questionnaire (IPAQ)-Short and Long Forms* (Online). IPAQ. http://www.ipaq.ki.se/scoring.pdf [November 2005].

18. **Juul-Kristensen B, Kadefors R, Hansen K, Bystrom P, Sandsjo L, Sjogaard G.** Clinical signs and physical function in neck and upper extremities among elderly female computer users: the NEW study. *Eur J Appl Physiol* 96: 136–145, 2006.

19. **Kadi F, Charifi N, Henriksson J.** The number of satellite cells in slow and fast fibres from human vastus lateralis muscle. *Histochem Cell Biol* 126: 83–87, 2006.

20. **Kadi F, Eriksson A, Holmner S, Butler-Browne GS, Thornell LE.** Cellular adaptation of the trapezius muscle in strength-trained athletes. *Histochem Cell Biol* 111: 189–195, 1999.

21. **Kadi F, Hagg G, Hakansson R, Holmner S, Butler-Browne GS, Thornell LE.** Structural changes in male trapezius muscle with work-related myalgia. *Acta Neuropathol (Berl)* 95: 352–360, 1998.

22. **Kadi F, Schjerling P, Andersen LL, Charifi N, Madsen JL, Christensen LR, Andersen JL.** The effects of heavy resistance training and detraining on satellite cells in human skeletal muscles. *J Physiol* 558: 1005–1012, 2004.

23. **Kadi F, Waling K, Ahlgren C, Sundelin G, Holmner S, Butler-Browne GS, Thornell LE.** Pathological mechanisms implicated in localized female trapezius myalgia. *Pain* 78: 191–196, 1998.

24. **Larsson B, Bjork J, Kadi F, Lindman R, Gerdle B.** Blood supply and oxidative metabolism in muscle biopsies of female cleaners with and without myalgia. *Clin J Pain* 20: 440–446, 2004.

25. **Lindström M, Thornell LE.** New multiple labelling method for improved satellite cell identification in human muscle: application to a cohort of power-lifters and sedentary men. *Histochem Cell Biol* 132: 141–157, 2009.

26. **Mackey AL, Bojsen-Moller J, Qvortrup K, Langberg H, Suetta C, Kalliokoski KK, Kjaer M, Magnusson SP.** Evidence of skeletal muscle damage following electrically stimulated isometric muscle contractions in humans. *J Appl Physiol* 105: 1620–1627, 2008.

27. **Mackey AL, Esmarck B, Kadi F, Koskinen SOA, Kongsgaard M, Sylvestersen A, Hansen JJ, Larsen G, Kjaer M.** Enhanced satellite cell proliferation with resistance training in elderly men and women. *Scand J Med Sci Sports* 17: 34–42, 2007.

28. **Mackey AL, Holm L, Reitelseder S, Pedersen TG, Doessing S, Kadi F, Kjaer M.** Myogenic response of human skeletal muscle to 12 weeks of resistance training at light loading intensity. *Scand J Med Sci Sports.* doi: 10.1111/j.1600-0838.2010.01178.

29. **Mackey AL, Kjaer M, Charifi N, Henriksson J, Bojsen-Moller J, Holm L, Kadi F.** Assessment of satellite cell number and activity status in human skeletal muscle biopsies. *Muscle Nerve* 40: 455–465, 2009.

30. **Mackey AL, Kjaer M, Dandanell S, Mikkelsen KH, Holm L, Dossing S, Kadi F, Koskinen SOA, Jensen CH, Schroder HD, Langberg H.** The influence of anti-inflammatory medication on exercise-induced myogenic precursor cell responses in humans. *J Appl Physiol* 103: 425–431, 2007.

31. **Mauro A.** Satellite cell of skeletal muscle fibers. *J Biophys Biochem Cytol* 9: 493–495, 1961.

32. **Mikkelsen UR, Langberg H, Helmark IC, Skovgaard D, Andersen LL, Kjaer M, Mackey AL.** Region specific prostaglandin blockade inhibits satellite cell proliferation in human skeletal muscle following eccentric exercise. *J Appl Physiol* 107: 1600–1611, 2009.

33. **Murphy JL, Blakely EL, Schaefer AM, He L, Wyrick P, Haller RG, Taylor RW, Turnbull DM, Taivassalo T.** Resistance training in patients with single, large-scale deletions of mitochondrial DNA. *Brain* 131: 2832–2840, 2008.

34. **Paulsen G, Egner IM, Drange M, Langberg H, Benestad HB, Fjeld JG, Hallen J, Raastad T.** A COX-2 inhibitor reduces muscle soreness, but does not influence recovery and adaptation after eccentric exercise. *Scand J Med Sci Sports* 20: e195–e207, 2010.

35. **Pedersen MT, Blangsted AK, Andersen LL, Jorgensen MB, Hansen EA, Sjogaard G.** The effect of worksite physical activity intervention on physical capacity, health, and productivity: a 1-year randomized controlled trial. *J Occup Environ Med* 51: 759–770, 2009.

36. **Petrella JK, Kim JS, Mayhew DL, Cross JM, Bamman MM.** Potent myofiber hypertrophy during resistance training in humans is associated with satellite cell-mediated myonuclear addition: a cluster analysis. *J Appl Physiol* 104: 1736–1742, 2008.

37. **Przybyla B, Gurley C, Harvey JF, Bearden E, Kortebein P, Evans WJ, Sullivan DH, Peterson CA, Dennis RA.** Aging alters macrophage properties in human skeletal muscle both at rest and in response to acute resistance exercise. *Exp Gerontol* 41: 320–327, 2006.

38. **Reimann J, Brimah K, Schroder R, Wernig A, Beauchamp JR, Partridge TA.** Pax7 distribution in human skeletal muscle biopsies and myogenic tissue cultures. *Cell Tissue Res* 315: 233–242, 2004.

39. **Renault V, Piron-Hamelin G, Forestier C, DiDonna S, Decary S, Hentati F, Saillant G, Butler-Browne GS, Mouly V.** Skeletal muscle regeneration and the mitotic clock. *Exp Gerontol* 35: 711–719, 2000.

40. **Shen W, Li Y, Zhu J, Schwendener R, Huard J.** Interaction between macrophages, TGF-beta1, and the COX-2 pathway during the inflammatory phase of skeletal muscle healing after injury. *J Cell Physiol* 214: 405–412, 2008.

41. **Sjogaard G, Rosendal L, Kristiansen J, Blangsted AK, Skotte J, Larsson B, Gerdle B, Saltin B, Sogaard K.** Muscle oxygenation and glycolysis in females with trapezius myalgia during stress and repetitive work using microdialysis and NIRS. *Eur J Appl Physiol* 108: 657–669, 2010.

42. **Thornell LE, Lindström M, Renault V, Klein A, Mouly V, Ansved T, Butler-Browne G, Furling D.** Satellite cell dysfunction contributes to the progressive muscle atrophy in myotonic dystrophy type 1. *Neuropathol Appl Neurobiol* 35: 603–613, 2009.

43. **Tidball JG, Wehling-Henricks M.** Macrophages promote muscle membrane repair and muscle fibre growth and regeneration during modified muscle loading in mice in vivo. *J Physiol* 578: 327–336, 2007.

44. **Verdijk LB, Koopman R, Schaart G, Meijer K, Savelberg HH, van Loon LJ.** Satellite cell content is specifically reduced in type II skeletal muscle fibers in the elderly. *Am J Physiol Endocrinol Metab* 292: E151–E157, 2007.

45. **Verney J, Kadi F, Charifi N, Feasson L, Saafi MA, Castells J, Piehl-Aulin K, Denis C.** Effects of combined lower body endurance and upper body resistance training on the satellite cell pool in elderly subjects. *Muscle Nerve* 38: 1147–1154, 2008.

46. **Visser B, van Dieen JH.** Pathophysiology of upper extremity muscle disorders. *J Electromyogr Kinesiol* 16: 1–16, 2006.

47. **Vogel H, Zamecnik J.** Diagnostic immunohistology of muscle diseases. *J Neuropathol Exp Neurol* 64: 181–193, 2005.

48. **Wakayama Y, Schotland DL, Bonilla E, Orecchio E.** Quantitative ultrastructural study of muscle satellite cells in Duchenne dystrophy. *Neurology* 29: 401–407, 1979.

49. **Winter A, Bornemann A.** NCAM, vimentin and neonatal myosin heavy chain expression in human muscle diseases. *Neuropathol Appl Neurobiol* 25: 417–424, 1999.