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ABSTRACT

Aim: To examine changes in lean mass during hospitalization in geriatric patients and the effect of muscle activation by neuromuscular electrical stimulation. **Methods:** Thirteen patients (69-94 years) at a geriatric ward completed tests at hospital admission (day 2-3) and discharge (day 8-10). One leg received daily stimulation of the knee extensors while the other leg served as a control leg. Lean mass was evaluated by DEXA scans and muscle thickness by ultrasound scans. Muscle biopsies were collected from both legs at admission and discharge in 9 patients and analyzed for fibre size, satellite cell number and activation and expression of genes associated with muscle protein synthesis and breakdown, connective tissue and cellular stress. **Results:** The relative decline in leg lean mass and midthigh region lean mass was larger in the control (-2.8±1.5%) vs. the stimulated leg (-0.5±1.4%, $p<0.05$). Although there were no changes in fibre size or satellite cell number, the mRNA data revealed that, compared to control, the stimulation resulted in a downregulation of Myostatin ($p<0.05$) and a similar trend for MAFbx ($p=0.099$), together with an upregulation of Collagen I ($p<0.001$), TenascinC ($p<0.001$), CD68 ($p<0.01$) and Ki67 ($p<0.05$) mRNA. **Conclusion:** These findings demonstrate a moderate decline in leg lean mass during a hospital stay in geriatric patients, while leg lean mass was preserved with daily neuromuscular electrical muscle activation. At the cellular level the stimulation had a clear influence on suppression of atrophy signaling pathways in parallel with a stimulation of connective tissue and cellular remodeling processes.

Keywords: Muscle Atrophy, Muscle Activation, Neuromuscular Electrical Stimulation, Satellite Cells, Gene Expression.

Introduction

Age-related loss of muscle mass is associated with poor mobility, loss of independency, increased hospitalization and increased mortality (1). In the present study the term “geriatric” is used for old (≥65 year) individuals admitted to a geriatric hospital ward, while the term “old” is used for healthy non-hospitalized individuals (≥60 years). The number of non-consecutive days spent in a hospital during a year is associated with loss of muscle mass and strength in old individuals (2), and it has therefore been suggested that the age-related loss of muscle mass is accelerated during shorter periods of illness or hospitalization followed by incomplete recovery (3, 4). Whether hospitalization associated loss of muscle mass actually occurs during the hospital stay in patients admitted to a geriatric ward has, however, only been sparsely investigated (5, 6). In healthy old individuals it has been shown that ageing is negatively associated with recovery of muscle mass after an experimental model of disuse atrophy (7). These authors furthermore noted a relationship between muscle re-growth and satellite cell (SC) proliferation during rehabilitation, with a reduced SC response in healthy old compared to healthy young subjects (7). The SCs are essential for muscle fibre regeneration and repair, and are the source of new myonuclei during muscle fibre hypertrophy (8), thus preservation and activation of SCs during incidents of short-term atrophy may be beneficial for subsequent recovery. It is, however, debated whether SCs are lost during short-term muscle fiber atrophy (9, 10).

Several factors may contribute to loss of muscle mass during hospitalization in geriatric patients; i.e. nutritional status, activity level and systemic factors related to their medical condition (3, 4, 11). In healthy old individuals, various models of inactivity can induce muscle atrophy (12–14), but this can be prevented with bouts of heavy resistance training (14) or neuromuscular electrical

stimulation (15). In contrast, geriatric patients are not completely inactive, but are often characterized by a low activity level (16). In addition, endocrine and inflammatory responses in these patients may result in further elevated muscle protein breakdown (3, 4, 11), potentially accelerating loss of muscle mass compared to models of disuse atrophy in healthy individuals. The literature is sparse regarding both loss of muscle mass (5, 6) and the effect of daily bouts of muscle activation during hospitalization in geriatric patients (5), and to our knowledge this has not been investigated at the cellular level in muscle biopsies. We have previously demonstrated a positive effect on leg lean mass with a unilateral heavy resistance training model in geriatric patients (5), but in that study we did not observe a decline in lean mass in the untrained leg. We speculated if there was a cross-education effect from the unilateral heavy resistance training intervention, as lean mass in an immobilized limb may be preserved by resistance training of the non-immobilized limb (17). In contrast, we are not aware of studies reporting a cross-education effect on muscle mass in the non-stimulated limb after unilateral neuromuscular electrical stimulation (E-Stim). E-Stim may therefore serve as a more suitable unilateral study design in order to examine both the loss of leg lean mass during a hospital stay and the counteracting effects of daily bouts of muscle activation. Furthermore, heavy resistance training may have some limitations in a geriatric patient group, particularly in those with poor mobility and motivation or in patients who are tired, dizzy or in pain. In contrast, E-Stim can be applied while the patients are lying in their bed, and can potentially induce forceful muscle contractions if tolerated by the patients. Although, E-Stim have shown promising effects on leg lean mass during bedrest in healthy old (15) and on muscle fibre size in critically ill comatose patients (18), it has not been investigated whether E-Stim is an effective method to preserve muscle mass in geriatric patients.

The aim of the present study was therefore to investigate the effect of a hospital stay either with or without daily sessions of E-Stim induced muscle contractions on changes in muscle mass, muscle fibre size, SC content and muscle gene expression levels in geriatric patients. It was hypothesized that E-Stim would preserve lean mass, muscle fibre size and SC content, increase the number of activated SCs, downregulate genes associated with muscle proteolysis and upregulate genes associated with muscle protein synthesis and cellular stress.

Methods

Ethical approval

The study was approved by the Research Ethics Committees of the Capital Region of Denmark (H-15005016) and conformed to the standards set by the Declaration of Helsinki, except for registration in a database. All patients signed a written informed consent agreement upon inclusion to the study.

Participants

The study was conducted in a 31 bed Geriatric ward at Bispebjerg Hospital, Capital region of Denmark, from August 2015 to July 2016. Patients admitted to this geriatric ward were ≥65 years old. During this period 805 patients were admitted to the department and screened for eligibility to participate in the study. After approval by the responsible medical doctor at the department, Danish speaking, non-terminal, non-isolated patients with an expected length of stay of ≥7 days were included if they were cognitively well-functioning (no history of dementia or Alzheimer's disease), medically stable enough to participate, physically able to complete the test-battery, did not have a medical condition known to accelerate loss of muscle mass (terminal

cancer, congestive heart failure, severe chronic obstructive pulmonary disease, HIV/AIDS), had similar function and size of the lower limbs, and did not have a pacemaker/ICD. Patients who were not eligible to, or declined to, participate in the muscle biopsy procedure were either offered to participate in a heavy resistance training study in the same department (5), or were included in the present study without the muscle biopsy procedure. A total of 17 patients were included in the study (6 males, 11 females). Four female patients did not complete the study because the test battery was too exhausting (n=2) or due to worsening of their medical condition not related to the study (n=2). Therefore, a total of 13 patients (6 males/7 females) completed the intervention and muscle biopsies were collected in 9 of these patients (5 males/4 females). Two patients were admitted directly to the geriatric ward, while 11 patients were initially admitted to an acute care facility for 1 day (n=6) or 2 days (n=5), before being transferred to the geriatric ward. Anthropometric data of the included patients are presented in Table 1. Most of the patients were admitted with more than one diagnosis, the most frequently occurring being: fall incident (n=4), lower back pain (n=5), infection (n=4), pneumonia (n=3), dehydration (n=2) and dizziness (n=2).

Study Design

At the first day of admission to the department (Day 1), eligible patients received oral and written information of the study and signed an informed consent the following day after approval by the responsible medical doctor. A test-battery was completed at admission (Pre, Day 2) and discharge (Post, Day 8-10). The test-order was consistently repeated, starting with muscle scans followed by muscle biopsies and finally tests of muscle function and general function. The intervention consisted of a daily E-Stim-session of the m.vastus lateralis (VL) and m.vastus

medialis (VM) muscles of one leg (E-Stim), while the other leg served as a control leg (CON). E-Stim was randomly assigned to the strongest or weakest leg, starting with familiarization to the E-Stim-protocol at the end of the Pre-test day. The following days (7 days a week), one daily E-Stim-session was completed, until and including the day before the Post-test (day of discharge or no later than day 10 of the hospital stay). No adverse effects of the E-Stim protocol were observed, and no patients dropped out of the study because of the E-Stim sessions. There was a 100% compliance to the E-Stim protocol, with an average of 6.6±1.0 days and E-Stim sessions between the Pre and Post-test. Activity level was recorded as the percent time spent inactive (lying down/sitting down) during the hospital stay with an ActivPAL accelerometer (PAL Technologies, Glasgow, Scotland) attached to the front of the thigh. A reliable step count could not be recorded in these patients.

E-Stim intervention

One daily 30-minute E-Stim session was applied to the knee extensor muscles (VL and VM) of the E-Stim leg, with a CefarCompex mi-Theta 600 Muscle stimulator (DJO Nordic, Malmoe, Sweden). A pillow was placed under the knee of the stimulated leg, ensuring a slightly bended knee (50° knee angle, 0° = fully straight leg). A 10x5cm self-adhesive electrode was placed on the midline of the anterior part of the thigh, 5 cm distal to the inguinal crease. One 5x5cm electrode was placed at the distal part of the VL and VM respectively after a motor point search, and the position of the electrodes was marked for accurate relocation on following days. The stimulation protocol was adopted from two recent studies showing positive effect of E-Stim during immobilization (19) and in comatose patients (18). Briefly, the protocol consisted of a 5-minute warm up/cool down sequence (5Hz, 250μs) and a 30-minute stimulation sequence (5

second on / 10 second off, 100Hz, 400µs, 0-120mA) with a 0.75 second rise/fall and a 3.5 second contraction phase. The patients were instructed not to co-contract the muscles during the stimulation. The stimulation intensity was gradually increased during every session and from session to session, as tolerated by the patient. The stimulation intensity of the VL in the first session started at 42±19 mA (mean±SD) and increased gradually to 61±21 mA at the end of the session. Stimulation intensity in the last session started at 68±26 mA and increased gradually to 89±24 mA at the end of the last session. When the stimulation resulted in a heel lift from the mattress, the leg was held down to the mattress by placing a hand on the ankle or with a strap during forceful contractions.

Measurements

Functional performance

Whole body functional testing included a test for mobility (DeMorton Mobility Index, DEMMI), lower limb strength and endurance (30 second chair stand test, 30-s CST) and gait speed (4 meter gait speed test, 4m-GST), performed according to the guidelines (20–22).

Muscle power and muscle strength

Lower limb extension power (muscle power) was tested in a Nottingham PowerRig (Queen's Medical Centre, Nottingham University, UK). After familiarization, 5 maximal attempts were performed with the same leg, followed by additional trials until the maximal score was followed by 2 lower attempts.

Maximal isometric knee extension torque (maximal voluntary contraction; MVC) was measured in a customized chair as described Norheim et al. (5). After a warmup trial, a total of three MVCs (6 seconds per trial) were performed for each leg, alternating between the legs with 2 minutes rest between attempts with the same leg. The maximal force (Newton) for each leg was recorded from the best trial, and the MVC (Nm) was calculated using the moment arm (m) from the dynamometer to the rotational axis of the knee.

Handgrip strength was measured with a Jamar® hydraulic hand dynamometer at admission only, serving as a descriptive measure of the patients. The patients performed a minimum of 3 attempts with both hands, until no further improvements were registered.

Lean mass (DEXA)

A whole-body dual energy x-ray absorptiometry scan (DEXA) was performed in a Lunar DPX-IQ DEXA scanner (GE Healthcare, Chalfont St. Giles, UK), and analyzed with the standard software package (Lunar iDXA Forma enCORE vs.15) by an investigator blinded to patient ID, time and treatment. Patients were positioned in the scanner with 10 cm distance between the heels to separate the lower limbs in the subsequent analysis. Lean mass was defined as soft tissue without fat mass and bone. Segmentation of body parts was performed according to the manufacturer's guidelines, and leg lean mass data was extracted from this analysis. The skeletal muscle index (SMI = appendicular lean mass/height^2) was calculated and compared to cut-off levels for sarcopenia (1) for men (<7.26kg/m^2) and women (<5.45kg/m^2). Midthigh region lean mass was defined as lean mass in a 4 cm region at the midthigh level with a method slightly modified from Norheim et al. (5). The midthigh region of the thigh was included as it may be the

region where the largest relative changes in muscle size after inactivity occurs (23), and because it corresponds to the site where muscle biopsies were collected. Briefly, with the Lunar iDXA software, a stack of 4 cm thick boxes was placed over the thigh, starting at the most distal part of the lateral condyle of the femur. Femur length was measured in the Lunar iDXA software and 50% femur length was defined as half the length from the most proximal point of the greater trochanter of the femur to the tibia plateau. The box covering the position corresponding to 50% femur length was included for analysis of midthigh region lean mass.

Muscle thickness (US)

Ultrasound (US) scans of the knee-extensor muscle thickness (VL; m.vastus lateralis + VI; m.vastus intermedius) in the transversal plane of the thigh at 50% femur length were included to complement the DEXA scans. A detailed description of the method for recording the US images has been published elsewhere (24). Briefly, the thickness of the VL and VI was measured by an experienced operator with B-mode ultra sound (GE Medical Systems. LogiQe), while the patients were seated on a chair with 90° flexion in the hip and knee joint. The deep aponeurosis separating VL and VI often had a wavy shape, preventing a reliable measurement of VL thickness; therefore, the combined thickness of VL+VI was measured. A total of 7 images were recorded for each measurement, the images were analyzed 5 times with ImageJ (version 1.51n; National Institute of Health; USA), and the highest and lowest value was discarded before calculating the mean VL+VI thickness for each image. The 2 highest and 2 lowest scoring images where then discarded, before VL+VI thickness was calculated as the mean of the remaining 3 images.

Muscle biopsies

A total of 4 muscle biopsies were taken, 1 Pre-biopsy and 1 Post-biopsy from the VL in both legs. Biopsies were taken with 2-3 cm between incision sites, randomized with respect to proximal/distal position between Pre- and Post-biopsies. Biopsies were taken after muscle scans and the procedure was followed by 1-2 hours of rest before the functional tests. The Post biopsies were taken approximately 20 hours after the last E-Stim session due to logistical reasons. Briefly, at the morning conferences it was decided by the medical team if a patient was discharged from the department on the same day, and therefore it was not possible to plan ahead before the Post-test. The E-Stim sessions were performed every day in the afternoon, being the time of day where it was most likely to get access to the patients, resulting in ~20 hours from the last E-Stim to the Post-test biopsies. After making an incision in the skin, biopsies were collected with the Bergström percutaneous needle biopsy technique and manual suction, under local anesthetic (1% lidocaine). The specimen was carefully aligned, embedded in Tissue-Tek® and frozen in isopentane precooled by liquid nitrogen. The embedded samples were stored at -80°C until all biopsies were collected. 10 µm thick sections were then cut in a cryostat at -20°C, placed on glass slides and stored at -80°C.

Immunohistochemistry

Three staining protocols were performed on separate glass slides for the analysis of: Staining 1) Muscle fibre cross-sectional area (CSA) and fibre type; Staining 2) Satellite cells and fibre type; Staining 3) Proliferating satellite cells. Detailed description of the staining protocols can be found in the Supplemental Digital Content (see Document, SDC 1, Methods), primary and

secondary antibodies are listed in the Supplemental Table S1 (see Table, SDC 2, primary and secondary antibodies for immunohistochemistry).

Image acquisition and analysis

All image analysis was performed in ImageJ (version 1.51n; National Institute of Health; USA) by an investigator blinded to subject ID, treatment and time point. Fibre cross-sectional area (CSA) was analyzed with a semi-automatic macro in ImageJ, according to methods published elsewhere (25). A detailed description of all image analyses can be found in the Supplemental Digital Content File (see Document, SDC 1, Methods).

On average 249±130 (range 89-696) type I fibres and 235±145 (range 15-669) type II fibres were analyzed for muscle fibre CSA. SCs were analyzed in 518±292 (range 109-1430) type I fibres and 550±454 (range 30-2213) type II fibres. One patient had very few type II fibres in the Post biopsies, with 15/30 (Post CON) and 52/60 (Post E-Stim) type II fibres included in the CSA/SC analysis respectively. In addition, fibre CSA was analyzed in 89 type I fibres in one biopsy and in 67 type II fibres in one biopsy, while all other fibre CSA and SC analyses included more than 100 fibres for each fibre type. All regions with muscle tissue (7.2±2.9mm^2), excluding large bands of connective tissue, were analyzed for proliferating cells ($Ki67^+$ cells) as well as proliferating SCs ($Ki67^+/Pax7^+$ cells). The number of $Ki67^+$ cells per biopsy was expressed relative to mm^2 muscle tissue.

Gene expression (mRNA)

Detailed description of the method for RNA extraction and real time RT PCR is available in the Supplemental Digital Content File (see Document, SDC 1, Methods). Primers for real time RT PCR are listed in the Supplemental Table S2 (see Table, SDC 3, primers for real-time PCR).

Statistics

Anthropometric data, i.e. changes in whole body fat mass and whole-body lean mass, were analyzed with a 2-tailed t-test for pairwise comparison. Functional test-scores (4m-GST, DEMMI-score and 30-s CST) and the number of proliferating cells in muscle tissue sections were analyzed with the non-parametric Wilcoxon Signed Rank test. Unilateral muscle function (lower limb extension power and maximal knee extensor strength), leg lean mass, midthigh region lean mass, muscle thickness, muscle fibre CSA and SC content was analyzed with a mixed 2-way ANOVA (time [Pre vs. Post, repeated measures factor], treatment [E-Stim vs. CON, group factor]), and the Holm-Sidak method was used for post hoc test of significant time x treatment interactions. In addition, data from scans of muscle mass where further analyzed for the difference between the individual relative changes in the E-Stim and CON leg with a 2-tailed t-test for pairwise comparison. mRNA data were log transformed and analyzed with a 2-tailed t-test for pairwise comparison of changes from pre to post within each leg as well as for the difference in changes between the legs. Data are presented as means ± SD, unless otherwise stated. A P-value <0.05 was considered statistically significant. All statistical analyses were performed with SigmaPlot vs 13.0 (Systat Software Inc., San Jose, CA).

Results

Descriptive data

Anthropometric data, functional test-scores and activity level during the hospital stay are presented in Table 1. Six of the patients (4 men, 2 women) had an SMI below the cut-off level for sarcopenia.

Functional Tests, Muscle Power and Maximal Quadriceps Strength.

Four of the patients were not able to rise from a chair without using their arms (30-s CST = 0 repetitions). Three patients did not use a walking aid, one patient walked with a cane, seven patients used a rollator walker and two patients used a high walking table. The average muscle power at Pre was 84±51 watts in the men (1.2±0.5 watts/kg) and 59±36 watts in the women (1.0±0.5 watts/kg). For comparison, leg extension power in 75-79 year old healthy individuals is considerably higher, i.e. 148±37 watts (2.3 watts/kg) in healthy old men and 80±27 watts (1.5 watts/kg) in healthy old women (26). There were no significant changes in the whole-body functional tests during the hospitalization (DEMMI-score, 30-s CST, 4m-GST, P =0.32-0.78, Table 2). In the unilateral tests of muscle power and maximal knee extensor torque there were no significant effect of time (P =0.17-0.66), treatment (P =0.25-0.68) or time x treatment interaction (P =0.10-0.77, Table 2) in the E-Stim and CON leg.

DEXA scans and Ultrasound scans

The mixed 2-way ANOVA analysis revealed a significant time x treatment interaction for leg lean mass (P <0.05), with a significant lower leg lean mass in the CON leg compared to the E-Stim leg after the intervention (-0.18±0.24kg, P <0.05, Table 2). In addition, midthigh region

lean mass showed a strong trend for a time x treatment interaction (P =0.06, Table 2). An explorative post hoc test revealed a significantly lower midthigh region lean mass in the CON leg vs. the E-Stim leg at post (P <0.05). There was no significant time x treatment interaction (P =0.22) for VL+VI thickness measured by ultra sound (Table 2). The individual relative changes in leg lean mass from Pre to Post, were significantly different between the E-Stim leg (-0.5±5.0%) and the CON leg (-2.8±5.3%, P <0.05, Figure 1). In addition, the individual relative changes in midthigh region lean mass was significantly different between the E-Stim (-0.7±4.6%) and the CON leg (-2.4±5.0%, P <0.05, Figure 1). The individual relative changes in VL+VI thickness were not significantly different between the E-Stim leg vs. the CON leg (P =0.33, Figure 1). There was no significant change in whole body lean mass (-0.34±1.49kg, P =0.43, Table 2), while whole body fat mass was reduced from Pre to Post (-0.35±0.45kg, P <0.05, Table 2).

Muscle fibre size, satellite cells and proliferating cells

At the cellular level there were no significant changes in muscle fibre size with hospitalization and E-Stim, as there were no main effects of time or treatment (P =0.21-0.95) and no time x treatment interaction for type I fibre CSA (P =0.54, Figure 2A) or type II fibre CSA (P =0.62, Figure 2B). In addition, SC-content was not affected by hospitalization and E-Stim, with no main effects of time or treatment (P =0.55-0.90) and no time x treatment interaction in type I fibres (P =0.44, Figure 3E) or type II fibres (P =0.72, Figure 3F). The patient who had very few type II fibres in the post biopsies (see Methods) is indicated in the figure (dotted lines) and removing this patient from the analysis did not change the outcome of the statistical tests. In the E-Stim leg there was a significant increase in the number of $Ki67^+$ cells per mm^2 muscle tissue from Pre to

Post ($p<0.05$, Figure 3K), while no changes were found in the prevalence of $Ki67^+$ cells/mm^2 muscle tissue in the CON leg ($p=0.13$, Figure 3K). Overall there were few $Ki67^+/Pax7^+$ cells in the biopsies, with five $Ki67^+/Pax7^+$ cells in the total 390 $Ki67^+$ cells identified. There was one $Ki67^+/Pax7^+$ cell in one Post E-Stim biopsy, and two $Ki67^+/Pax7^+$ cells in two Pre biopsies from the CON leg.

mRNA

Hospitalization did not affect gene expression in most of the genes measured in the CON leg except for a significant downregulation in MAFbx (P <0.05) and a trend for a downregulation in MURF1 (P =0.088, Figure 4A). In the E-Stim leg there was also a significant downregulation in MAFbx (P <0.05), with a trend for a larger downregulation compared to the CON leg (P =0.099), and MURF1 (P <0.05) was significantly downregulated (Figure 4A). There was furthermore a significant difference between the E-Stim and CON leg in the relative changes in myostatin gene expression (P <0.05, Figure 4A). Gene expression of Collagen 1 (P <0.001), TenascinC (P <0.001), CD68 (P <0.01) and Ki67 (P <0.05) were significantly upregulated in the E-Stim leg only, there was a trend for a downregulation of HSP70 (P =0.07, Figure 4B) and there was a significant difference between the E-Stim and the CON leg (P <0.001-0.05) for all these genes, together with a trend for a difference between the E-Stim and the CON leg in p16 (P =0.095, Figure 4C). No significant changes were observed in gene expression of GAPDH, IGF-1Ea, IGF-1Ec, Myogenin, NCAM, TCF7L2 and TNF-α (Figure 4C+D).

Discussion

The present study is to our knowledge the first study to investigate the effects of hospitalization upon skeletal muscle either with or without daily sessions of E-Stim induced muscle activation in patients admitted to a geriatric ward. DEXA scans revealed a significant positive effect of E-Stim in preserving leg lean mass and midthigh region lean mass. Functional test scores and muscle function did not change during the study. Although E-Stim increased the prevalence of proliferating cells in muscle biopsies from a sub-group of 9 patients there were no changes in muscle fibre size, the size of the SC pool and the prevalence of activated SCs. However, compared to the CON leg, E-Stim resulted in overall favorable changes in gene expression associated with muscle fibre atrophy (myostatin, MURF1, MAFbx), extracellular matrix remodeling (Collagen 1, TenascinC) and markers of cell activity (CD68, Ki67), together demonstrating a positive response at the cellular level.

Muscle function and physical function

When indirectly assessed with interviews, 35-65% of old medical patients have experienced functional decline before admission to the hospital (27, 28), and more than 50% of the patients are discharged with a decline in their functional level compared to their pre-admission level (27, 28). However, several studies do not observe functional decline during hospitalization in various measures of muscle function and mobility (16, 29, 30), possibly reflecting recovery from the patient's medical condition. In line with these observations, we did not observe any decline in measures of muscle strength and power in the CON leg. In addition, there was no decline in whole body functional test-scores, but it should be noted that these tests were included for general descriptive purposes, while they are not suited for evaluation of a unilateral intervention

as they include bilateral leg muscle activation. Although longer duration E-Stim interventions can improve functional performance and muscle strength (31), there was no measurable effect of the E-Stim on these parameters in the present study. This was somewhat expected due to the short duration of the intervention in the present study, and in line with our previous results after heavy resistance training with a comparable study design (5).

Lean Mass

Although the number of days spent in a hospital during one year is related to a decline in both lean mass and strength in old individuals (2), it is not well explored whether this actually occurs during the hospital stay. Two studies in acutely admitted geriatric patients have reported no decline in either leg lean mass (5) or whole body potassium (6). In the present study, the relative decline in leg lean mass and midthigh region lean mass was significantly larger in the CON leg compared to the E-Stim leg (Figure 1), and the CON leg had significantly lower leg lean mass vs. the E-Stim leg at the end of the hospital stay (Table 2). These results suggest a modest loss of muscle mass in the lower extremities in these patients. Although many factors may contribute to hospital associated loss of muscle mass; i.e. activity level, nutritional status and systemic factors related to the medical illness (3, 4, 11), one daily session of E-Stim induced muscle contractions efficiently preserved lean mass in the present study. This is a very important observation, in line with our previous report of a positive effect of resistance training on lean mass in patients admitted to the same geriatric ward (5). Together, these data demonstrate that geriatric patients respond in a similar positive manner to muscle activation during hospitalization as healthy old individuals during a period of inactivity (14, 15).

Noteworthy, the ~2.4% greater relative decline in leg lean mass in the CON leg corresponds to a daily decline in leg lean mass of approximately 0.4% in the absence of electrical stimulation. Disuse atrophy models in healthy old individuals result in a 0.3-0.7% decline per day in quadriceps volume with lower limb immobilization (13, 32), a 0.6-0.8% decline per day in leg lean mass with bed rest (12, 15, 33, 34) and a ~0.3% decline per day in leg lean mass in a model of reduced activity where step count was reduced to 1500 steps per day (35). The ~0.4% decline per day in leg lean mass in the absence of E-Stim in the present study therefore seems to be within the range of what is observed in disuse atrophy models in healthy old individuals. However, it is important to emphasize that the present results may underestimate the average loss of muscle mass in these patients for several reasons. First of all, the present study design included a demanding test battery, and the included patients may have been a selected group of patients, as they were younger, had better functional test-scores and slightly higher activity level, compared to a larger group of patients included in an observational study the year before at the same department (16). For ethical and practical reasons, the patients were Pre-tested on their second day at the geriatric department, many of the patients had already spent ~1 day at the acute care ward plus they may have been sick and inactive for 1-2 days before arriving at the hospital, and we may therefore have missed the time-point where the rate of loss of muscle mass peaked. Furthermore, as the Post-test was performed 6.6±1.0 days after the Pre-test, the duration of the intervention was considerably shorter than the average length of stay at this department (~10 days) at the time of the study (30). Finally, it should be noted that changes in lean mass (DEXA) can also be affected simply by a change in hydration status, for example in patients who were dehydrated at the time of admission to the hospital and became rehydrated during the hospital stay. However, the unilateral within-subject design accounts for this regarding differences

between the legs, so these potential limitations do not negate our findings of a preserved lean mass with E-Stim in geriatric patients during a hospital stay.

At the cellular level

At the cellular level we hypothesized that E-Stim could preserve muscle fibre size, while we did not expect any significant muscle fibre hypertrophy within the short duration of this intervention. Due to the lack of a significant decline in muscle fibre size in the CON leg, this hypothesis could not be confirmed. The lack of significant muscle fibre atrophy contrasted with the ~2.5% decline in leg lean mass in the DEXA scans. Significant muscle fibre atrophy together with a ~4% decline in thigh lean mass has previously been reported in healthy old individuals after short-term bed rest (15), while others have failed to detect significant muscle fibre atrophy despite a ~3.5% decline in quadriceps CSA after 5 days of immobilization (19). The present data therefore probably reflect that changes in muscle fibre size were smaller than what could be detected with the muscle biopsy technique in these patients. Interestingly, the changes from Pre to Post in the expression of genes related to the proteolytic ubiquitin signaling pathway overall indicated a further downregulation of MURF1 and MAFbx in the E-Stim leg compared to the CON leg. In addition, E-Stim induced favorable changes in gene expression of myostatin, a member of the TGF-β family and a negative regulator of muscle growth, with a reduced expression in the E-Stim leg vs. the CON leg. Somewhat surprising, although bedrest and immobilization are often associated with elevated gene expression of MURF1, MAFbx and/or myostatin in healthy old individuals (7, 15, 34, 36), we observed a downregulation of MURF1 and MAFbx in the CON leg during the hospital stay. Elevated mRNA levels of MURF1 and MAFbx have previously been reported in critically ill patients compared to healthy controls (18, 37), and although the

patients in the present study may not be comparable to critically ill patients, the present data probably reflect that MURF1 and MAFbx mRNA levels were elevated in the geriatric patients at the time of admission to the department. The relative decline in gene expression from admission to discharge may therefore reflect that the patients had recovered from their illness during the hospital stay. Without a healthy age and gender-matched control group we are unfortunately not able to determine if MURF1 and MAFbx mRNA levels were elevated at the time of the Pre-biopsy. In addition, it is not clear from these data whether MURF1 and MAFbx mRNA levels had returned to a normal level during the hospital stay. It could be speculated that the changes were the result of an increased activity level during the hospitalization, but day-to-day activity recordings showed no difference from the first to the last day of the hospital stay. However, it cannot be ruled out that with the medical treatment and daily support from the care personnel at the geriatric ward, the patients were able to be more physically active at the geriatric department compared to the acute care ward and the days leading up to the hospital admission. E-Stim resulted in a further and possibly earlier downregulation of these genes, which may be an important mechanism for the preservation of muscle mass. In contrast, E-Stim only resulted in small and non-significant increases in gene expression of IGF-1Ea ($p=0.203$) and IGF-1Ec ($p=0.127$), members of the IGF-1 family that are believed to play an important role in muscle hypertrophy, satellite cell activation, proliferation and differentiation (38), and also exert an inhibiting effect on the ubiquitin pathway (39). Upregulation in these IGF-1 isoforms was reported 7 days after the last training session of a 9 week electrical stimulation protocol in healthy old individuals (31). In the present study we may have missed the time point for a peak increase in IGF-1 expression, but it is also possible that the intervention was too short to induce significant changes.

Immunohistochemical analysis of SC content revealed that SCs were not lost in the CON leg during hospitalization, and that E-Stim did not increase the SC content. It is important to emphasize that it is not fully clear whether SCs are lost during disuse atrophy (9, 10). Without a significant reduction in the SC pool in the CON leg in the present study, it is not possible to conclude whether E-Stim could have preserved the SC pool in these patients. E-Stim could in addition be a potent stimulus for SC activation and proliferation, potentially elevating the SC-content of the stimulated muscles, which may be beneficial for subsequent rehabilitation (7). However, although gene expression of the cell-proliferation marker $Ki67^+$ increased with E-Stim, and there was a significant increase in the prevalence of $Ki67^+$ cells per mm^2 muscle, there was no increase in SC content and activated SCs ($Ki67^+/Pax7^+$ cells) in the E-Stim leg, and no increase in gene expression of myogenin and NCAM, suggesting a poor SC response to this intervention, at least at the time-point of the Post-biopsies. Our lab has previously used the Ki67 antibody to label activated SCs ($Pax7^+/Ki67^+$) cells after a severe muscle damaging protocol that resulted in a substantial increase in the number of SCs per fibre but only a modest increase in the proportion of $Ki67^+/Pax7^+$ cells 7 days after the intervention (40). Noteworthy, the increase in $Ki67^+/Pax7^+$ cells was not significant 48 hours after this muscle damaging protocol (40). In the present study, as part of the immunohistochemical staining, the Ki67 antibody was also tested on a sample with many $Pax7^+/Ki67^+$ cells from that study, confirming that the Ki67 antibody worked. It should be noted that the E-Stim intensity had to be increased gradually from session to session, as the tolerance to the stimulation gradually improved in the patients. The biopsies were taken in the deep portion of VL and it is likely that it took some days before the E-Stim intensity had reached a level high enough to recruit muscle fibres in this area. However, the mRNA data strongly suggest that muscle fibres in the biopsied region had been recruited during

the stimulation. The lack of SC-activation may furthermore be related to a blunted or delayed SC-responsiveness with aging (41). Therefore, although speculative, the present data cannot rule out the possibility that the E-Stim protocol could have resulted in activated SCs with a more prolonged intervention. The increase in Ki67 mRNA levels as well as $Ki67^{+}/Pax7^{-}$ cells per mm^2 demonstrates that other cell-types, for example immune cells, endothelial cells or fibroblasts were activated in response to the E-Stim. Based on the increase in CD68 mRNA we speculate that the activated cells were primarily macrophages, while the unchanged expression of TCF7L2 may indicate that the activated cells were not fibroblasts. However, it should be noted that although CD68 is frequently used as a marker of macrophages, it is also expressed in fibroblasts and endothelial cells (42), but it was not within the aim of the present study to quantify these cell types in detail. Overall, the gene expression data therefore seems to reflect an acute stress response and an increase in proliferating macrophages, further supported by the increased expression of Collagen I and TenascinC, genes related to extracellular matrix remodeling that are elevated after muscle damaging exercise (43). It should be noted that we did not register muscle soreness in the patients at any time during the study, and found no muscle fibres with a high density of central nuclei, a sign of myofibre necrosis and macrophage infiltration (44). In addition there was a trend for a downregulation of the expression of HSP70, a marker of cell stress that is upregulated as an early response to myofibre damage (43). The elevated gene expression of Collagen I and TenascinC may instead reflect a general response to the mechanical stress during forceful muscle contractions and a gradual adaptation of the myofibres and extracellular matrix to this type of stimulation.

Conclusion

The present study demonstrates the positive effects of E-Stim induced muscle contractions, preserving lean mass in geriatric patients during hospitalization, and resulting in favorable changes at the gene expression level with downregulation of genes associated with muscle protein breakdown. E-Stim may be the only option for muscle activation in patients who are not easily mobilized for various reasons, but similar effects may be achieved with structured functional training programs or with heavy resistance training in patients where this is feasible. Altogether the changes in gene expression in the present study demonstrate that E-Stim induced downregulation of genes involved in muscle atrophy, which may be related to the preservation of leg lean mass in the E-Stim leg.

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Conflict of interests

No conflicts of interest, financial or otherwise, are declared by the authors. The results of the present study do not constitute endorsement by ACSM. All authors declare that the results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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Figure Captions

Figure 1

Relative changes in leg lean mass and midthigh region lean mass measured with dual energy x-ray absorptiometry scan (DEXA) and the relative change in thickness of the thigh muscles (VL, m.vastus lateralis, VI, m.vastus intermedius) measured with ultra sound (US) scan. Bars illustrate the mean of the individual relative changes in the CON leg (grey bars) and the E-Stim leg (white bars). The individual relative changes are illustrated with white symbols (men) and filled symbols (women) for the CON leg (circles) and the E-Stim leg (triangles) in n=13 geriatric patients. *P <0.05 between the legs.

Figure 2

A-B: Muscle fibre size in type I fibres (A) and type II fibres (B), before (Pre) and after (Post) hospitalization in one leg receiving no treatment (CON) and the other leg receiving electrical stimulation (E-Stim). Bars illustrate mean fibre size for n=9 patients in the CON leg (grey bars) and the E-Stim leg (white bars) at Pre and Post. Individual data at Pre and Post are illustrated with white symbols (men) and filled symbols (women) for the CON leg (circles) and the E-Stim leg (triangles). The dotted lines indicate the patient with very few type II fibres in two of the biopsies. **C-E:** Confocal images for muscle fibre cross-sectional area analysis are shown in C-E. The colors are pseudo colors, showing laminin staining of basement membrane (white) and myosin heavy chain I staining of type I fibres (red). The vertical and horizontal yellow lines mark the overlap between neighboring tiles. Fibres included for analysis of fibre size are visualized in B and C as fibres delineated with a yellow line. Scale bars = 100µm

Figure 3

A-D: Widefield microscopy images for satellite cell analysis (A-D). The images show nuclei (DAPI, blue, A), satellite cells (pax7, green, B), together with basement membrane (laminin, red, C) and myosin heavy chain I (MHC-I, cyan, D). The white arrow (A-D) indicates a cell considered to be a satellite cell belonging to a type II fibre. The colors of laminin and MHC-I are pseudo colors. **E-F:** Number of satellite cells ($Pax7^+$ cells) per fibre in type I fibres (E) and type II fibres (F), before (Pre) and after (Post) hospitalization in one leg receiving no treatment (CON) and the other leg receiving electrical stimulation (E-Stim). Bars illustrate mean number of satellite cells per fibre for n=9 patients in the CON leg (grey bars) and the E-Stim leg (white bars) at Pre and Post. Individual data at Pre and Post are illustrated with white symbols (men) and filled symbols (women) for the CON leg (circles) and the E-Stim leg (triangles). The dotted lines (F) indicate the patient with very few type II fibres in two of the biopsies. **G-J:** Widefield microscopy images of staining for proliferating cells and satellite cells. The images show nuclei (DAPI, blue, G), proliferating cells (Ki67, red, H), satellite cells (pax7, green, I) and all channels combined (J). The white arrow indicates a Ki67 positive cell that is not pax7 positive (G, H, J). The yellow arrow indicates a pax7 positive cell that is not Ki67 positive (G, I, J). **K:** Number of Ki67 positive cells ($Ki67^+$) per muscle tissue cross-section (mm^2) in men (white symbols) and women (filled symbols) for the CON leg (circles) and the E-Stim leg (triangles). *P <0.05 vs. Pre. Scale bars = 100µm

Figure 4

Relative changes in gene expression levels (mRNA) in muscle biopsies in geriatric patients (n=9) collected in the same leg at admission and discharge from the hospital in the leg receiving no

special treatment (CON, grey bars) and the leg receiving electrical stimulation (E-Stim, white bars). RPLP0 mRNA was chosen as internal control (housekeeping gene), GAPDH mRNA was measured and normalized with RPLP0 to validate the use of RPLP0, with no difference for GAPDH, supporting the use of RPLP0 for normalization. Symbols in brackets () denotes a trend for significance at P =0.05-0.10. * denotes P <0.05 from Pre within the same leg, # denotes P <0.05 between the relative changes in the E-Stim and CON leg. Data are geometric means ± back-transformed SEM displayed on a logarithmic scale y axis (Log2)

List of Supplemental Digital Content

Supplemental Digital Content File.pdf - Methods

Supplemental Table S1.pdf - Primary and secondary antibodies for immunohistochemistry

Supplemental Table S2.pdf - Primers for real-time PCR

Figure 1

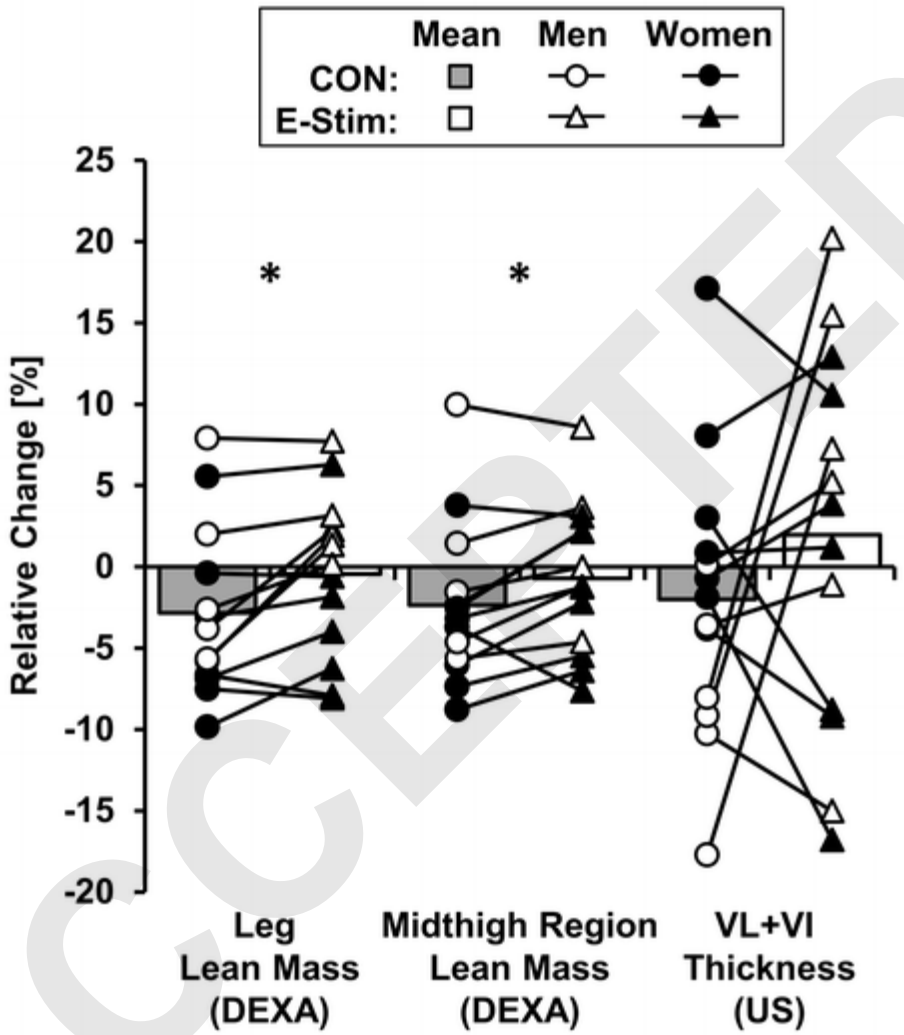

Figure 2

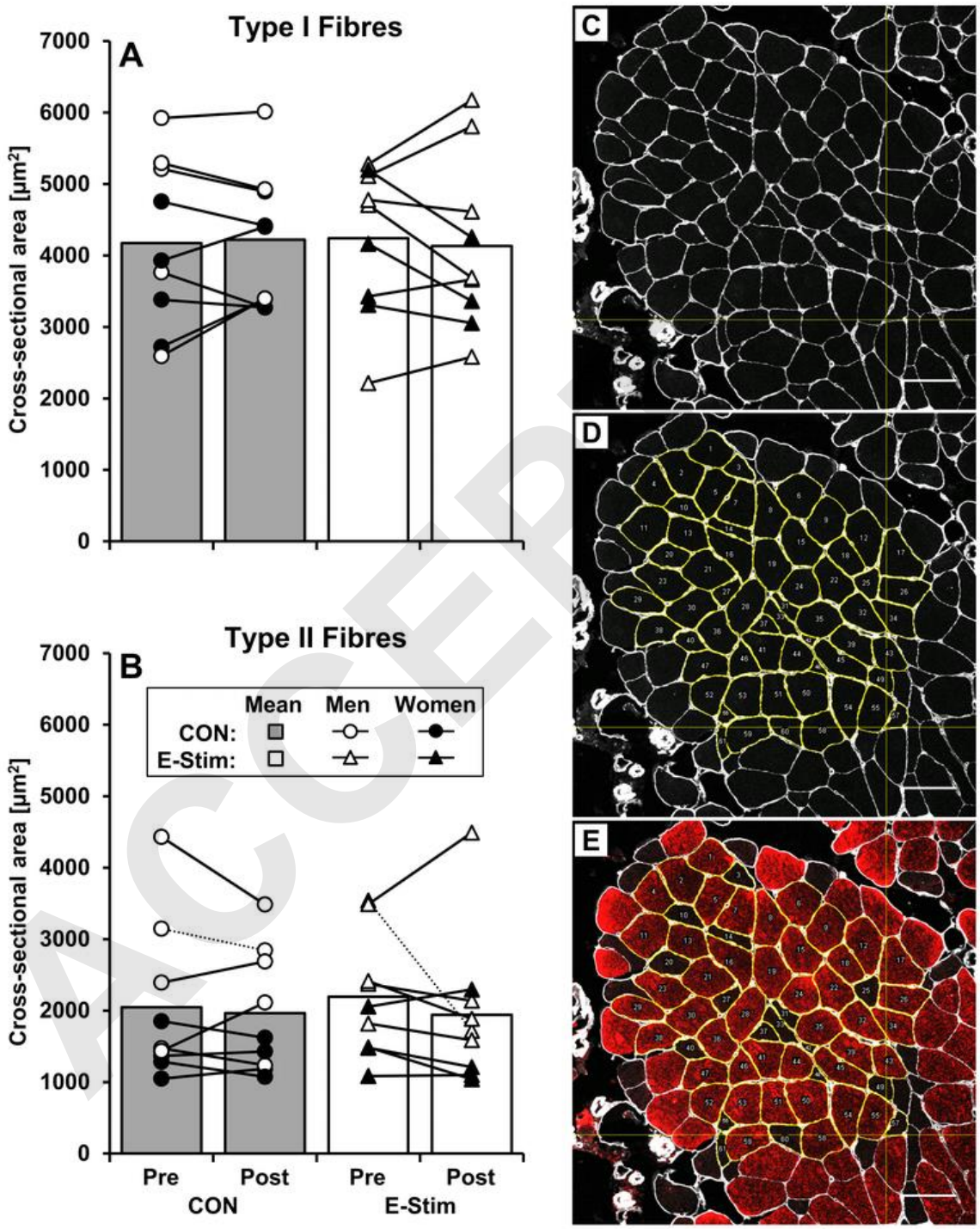

Figure 3

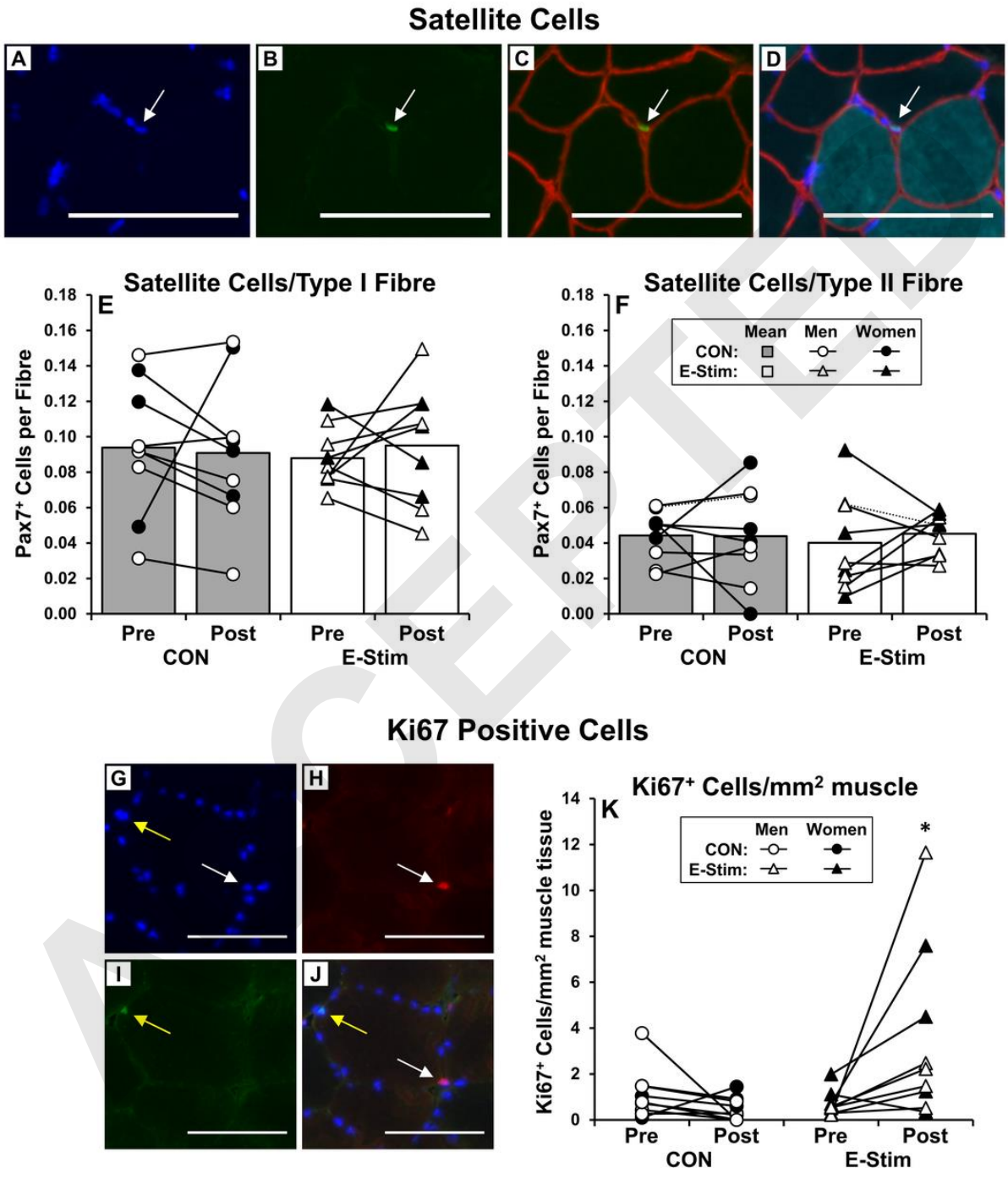

Figure 4

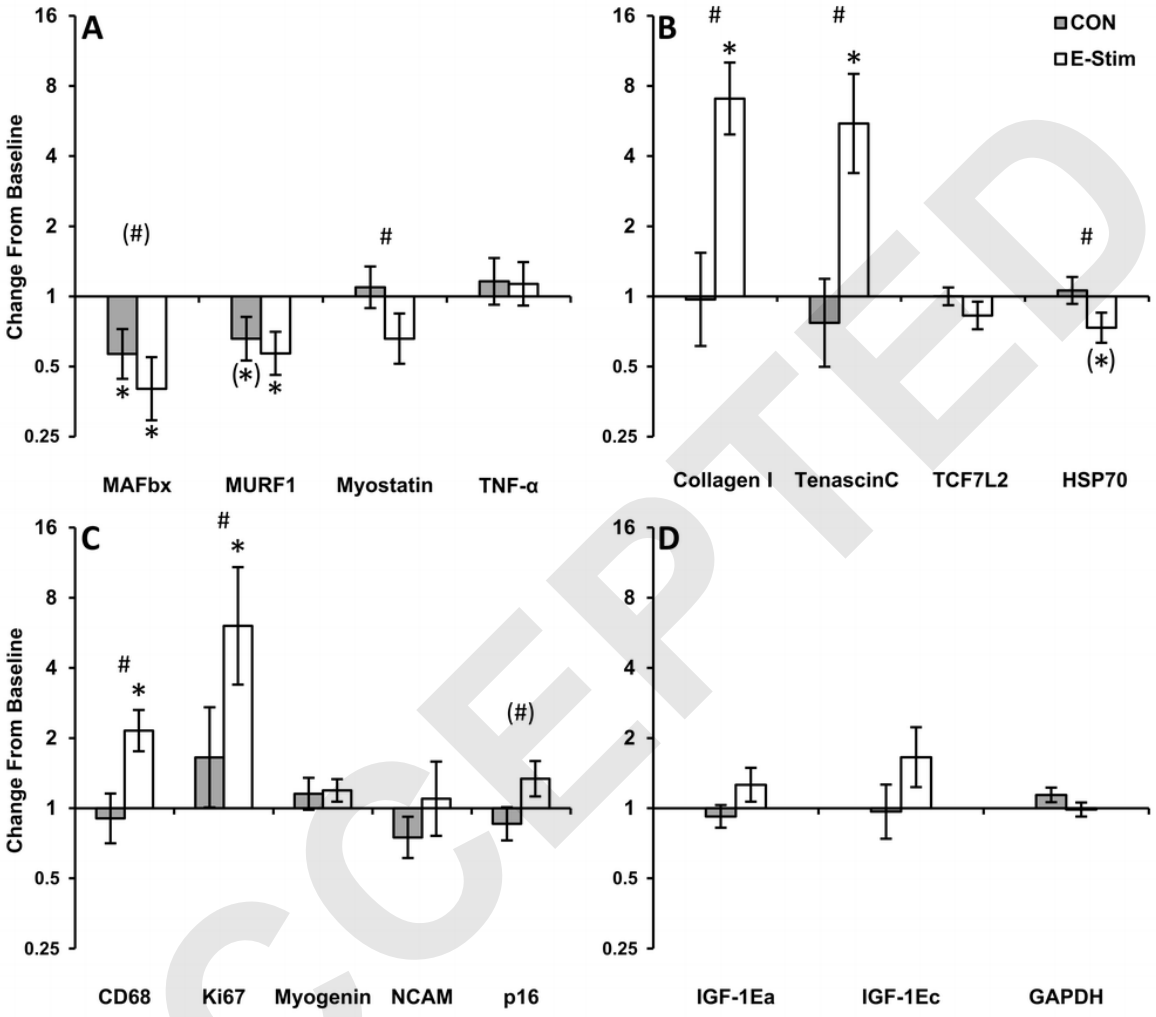

Table 1 Descriptive data of the patients at the time of admission

	All (n=13)	Men (n=6)	Women (n=7)
Age [years]	78 ± 8	74 ± 5	82 ± 8
Height [cm]	167 ± 10	170 ± 10	164 ± 9
Weight [kg]	63 ± 12	70 ± 10	56 ± 10
BMI [kg/m^2]	23 ± 4	24 ± 3	21 ± 4
SMI [kg/m^2]	6.3 ± 0.9	6.7 ± 1.1	5.9 ± 0.7
DEMMI-score [0-100]	57 [49:71]	67 [61:74]	53 [30:57]
30-s CST [number of reps]	5 [0:11]	8 [4:12]	1 [0:10]
4m GST [m/s]	0.7 [0.4:0.8][a]	0.7 [0.7:0.8]	0.5 [0.3:0.8][b]
Grip Strength [kg]	23 ± 7	28 ± 6	19 ± 6
Time spent inactive [%]	90 [87:96][a]	92 [88:97]	88 [75:94][b]

Baseline data for the 13 geriatric patients who completed the E-Stim intervention.

BMI=Body Mass Index,

SMI=Skeletal Muscle Index,

DEMMI=DeMorton Mobility index (0-100),

30-s CST=30 second chair stand test,

4m GST=4 meter gait speed test.

Due to missing data the number of patients assessed was: [a] n=12, [b] n=6. Data are means ± SD or medians [IQR].

Table 2 Functional test-scores, muscle function, lean mass and muscle thickness

		Pre	Post	Wilcoxon Signed Rank Test (p-value)	Main effect Time (p-value)	Main effect Treatment (p-value)	Time x Treatment Interaction (p-value)
DEMMI-score (0-100)[a]		60 [54:72]	62 [54:72]	0.31			
30-s CST (repetitions)[a]		7 [0:11]	7 [0:11]	0.81			
4m GST (m/s)[b]		0.7 [0.6:0.8]	0.7 [0.5:0.9]	0.21			
Muscle power (watt)	E-Stim	74 ± 44	68 ± 32		0.66	0.68	0.10
	CON	72 ± 47	71 ± 38				
MVC (Nm)	E-Stim	69 ± 24	71 ± 22		0.17	0.25	0.77
	CON	72 ± 22	75 ± 23				
		Pre	**Post**	**Paired t-test (p-value)**	**Main effect Time (p-value)**	**Main effect Treatment (p-value)**	**Time x Treatment Interaction (p-value)**
Total Lean Mass (DEXA, kg)		41.7 ± 4.7	41.3 ± 5.4	0.43			
Total Fat Mass (DEXA, kg)		19.5 ± 8.7	19.1 ± 8.5*	0.02			
Leg Lean Mass (DEXA, kg)	E-Stim	6.53 ± 0.72	6.53 ± 0.95		0.33	0.14	<0.05
	CON	6.51 ± 0.80	6.34 ± 0.96**				
Midthigh Region Lean Mass (DEXA, g)	E-Stim	377 ± 73	375 ± 79		0.40	<0.05	0.06
	CON	373 ± 74	366 ± 83				
VL+VI Thickness	E-Stim	2.41 ± 0.95	2.47 ± 1.06		0.87	0.61	0.22

(US, cm)	CON	2.43 ± 0.83	2.38 ± 0.85

Functional test-scores, muscle function and fat mass, lean mass and muscle thickness in n=13 geriatric patients Pre-tested at Day 2 and Post-tested at Day 8-10 during a hospital stay. One leg received electrical stimulation (E-Stim), while the other leg served as a control leg (CON). Functional tests included: DEMMI (DeMorton Mobility index, 0-100), 30-s CST (30 second chair stand test), 4m GST (4 meter gait speed test). Muscle power: Leg extension power, Nottingham PowerRig. MVC: Maximal voluntary contraction, i.e. knee extensor torque of the knee extensor muscles. DEXA: Dual energy x-ray absorptiometry scan. Lean Mass: Soft tissue without fat and bone. Midthigh Region: A 4cm thick slice transversally placed over the thigh at 50% femur length. VL: m.vastus lateralis. VI: m.vastus intermedius. US: Ultra sound scan.

[a] one patient did not complete the Post-test (n=12).

[b] two patients did not complete the test at Pre or Post (n=11).

* paired t-test $p<0.05$ vs. Pre.

** post-hoc test: significant difference vs. E-stim leg at Post $p<0.05$.

Data are means ± SD or medians [IQR].

Supplemental Digital Content File

Methods

Immunohistochemistry

Three staining protocols were applied:

Staining 1) Muscle fibre cross-sectional area (CSA) and fibre type;

Staining 2) Satellite cells and fibre type;

Staining 3) Proliferating satellite cells.

For all staining protocols, primary and secondary antibodies were diluted in 1% BSA in TBS (tris-buffered saline, tris-base 0.05 mol/l, sodium chloride 0.154 mol/l, pH 7.4-7.6), and sections were washed 2x5 minutes (staining 1) or 3x5 minutes (staining 2+3) in TBS between each protocol step. After removing sections from the freezer, sections were dried at room temperature and fixed in 4% PFA for 10 minutes (staining 1) or 5 minutes (staining 2+3), and incubated with primary antibodies overnight at 4 °C. The following day the appropriate secondary antibodies were diluted in 1% BSA in TBS and applied for 45 minutes at room temperature.

Primary and secondary antibodies are shown in Supplemental Table S1. In sections for confocal imaging (fibre CSA) nuclei were stained blue with Hoechst dye diluted in TBS (Hoechst 33342; 2.5µg/ml; H1399; Invitrogen), and slides were mounted with cover glasses in mounting medium (Molecular Probes ProLong Gold anti-fade reagent, cat. no. P36930). In sections for widefield microscopy (satellite cells and proliferating cells) DAPI in the mounting medium stained nuclei blue (Blue, Molecular Probes ProLong Gold anti-fade reagent, cat. no. P36931).

Image acquisition and analysis

All image analysis was performed in ImageJ (version 1.51n; National Institute of Health; USA) by an investigator blinded to subject ID, treatment and time point.

Staining 1, Muscle Fibre CSA: Representative images are available in Figure 2C-E. Sections stained for fibre CSA were digitally captured with a confocal laser scanning microscope (20x/0.8NA objective; pinhole = 3µm; LSM710, Carl Zeiss, Oberkochen, Germany). Images (716.8x716.8 µm/2048x2048 pixels) were captured as tiles (160 µm / 457 pixel overlap between tiles), using a semi-automated motorized function in the Zeiss ZEN software. The overlap between tiles was marked in ImageJ (Figure 1C), ensuring no fibres were excluded or analyzed twice, and tiles were randomly selected for analysis of fibre CSA. A detailed description of the method for fibre CSA analysis has furthermore been published elsewhere (1). Briefly, a macro developed for

ImageJ automatically delineated all fibres in a tile, followed by a manual approval step of all fibres included in the analysis (Figure 1D). Tiles were included for the CSA analysis until a minimum of 150 fibres of each fibre type (MHC-I positive/negative) had been analyzed, if possible. Regions with longitudinally cut fibres and fibres on the edges of the biopsy were not included in the fibre CSA analysis.

Staining 2, Satellite cells: Representative images are available in Figure 3A-D. Sections were imaged with a 10x/0.30NA objective on an Olympus BX51 microscope with a 0.5x camera (Olympus DP71, Olympus Deutschland GmbH, Hamburg, Germany) mounted on the microscope (image size 3964x2889µm or 3705x2700 pixels), controlled by the software Cell^F (Olympus Soft Imaging Solutions, GmbH, Münster, Germany). Images were recorded as tiles with manually adjusted overlap (approx. 150-300 pixel overlap) and automatically stitched together in ImageJ (plugins/stitching/deprecated/stitch directory with images (unknown configuration)). All fibres in the biopsies were included for the SC analysis, excluding damaged fibres and regions with longitudinally cut fibres.

Staining 3, Proliferating cells and Satellite Cells: Representative images are available in Figure 3G-J. $Ki67^+$ cells were counted in sections stained for proliferating cells and satellite cells, excluding large bands of connective tissue. The count was performed using the microscope eyepieces and a 20x/0.5NA objective, in the same microscope used for SC analysis. Images of the Ki67 channel were recorded with a 4x/0.1NA objective, and the cross-sectional area of included regions were analyzed manually in ImageJ.

Gene expression

RNA extraction: 100 cryo sections of 10 µm from the embedded muscle tissue were homogenized in 1 mL of TriReagent (Molecular Research Center, Cincinnati, OH, USA) containing five stainless steel balls of 2.3 mm in diameter (BioSpec Products, Bartlesville, Oklahoma, USA), and one silicon-carbide sharp particle of 1 mm (BioSpec Products), by shaking in a FastPrep®-24 instrument (MP Biomedicals, Illkirch, France) at speed level 4 for 15 s. Following homogenization, bromo-chloropropane was added to separate the samples into an aqueous and an organic phase. Following isolation of the aqueous phase, RNA was precipitated using isopropanol. The RNA pellet was then washed in ethanol and subsequently dissolved in 20 µL RNAse-free water. Total RNA concentrations and purity were determined by spectroscopy at 260, 280 and 240 nm. Good RNA integrity was ensured by gel electrophoresis.

Real-time RT-PCR: 500 ng total RNA was converted into cDNA in 20 µL using the OmniScript reverse transcriptase (Qiagen, California, USA) and 1 µM poly-dT (Invitrogen, Naerum, Denmark) according to the manufacture's protocol (Qiagen). For each target mRNA, 0.25 µL cDNA was amplified in a 25 µL SYBR Green polymerase chain reaction (PCR) containing 1 × Quantitect SYBR Green Master Mix (Qiagen) and 100 nM of each primer (for primers see Supplemental Table S2). The amplification was monitored real time using the MX3005P Real-time PCR machine (Stratagene, California, USA). The Ct values were related to a standard curve made with known concentrations of cloned PCR products or DNA oligonucleotides (UltramerTM oligos, Integrated DNA Technologies, Inc., Leuven, Belgium) with a DNA sequence corresponding to the sequence of the expected PCR product. The specificity of the PCR products was confirmed by melting curve analysis after amplification. RPLP0 mRNA was chosen as internal control. To validate this use, another unrelated "constitutive" mRNA, GAPDH, was measured and normalized with RPLP0. No difference was seen for GAPDH, supporting the use of RPLP0 for normalization.

References

1. Karlsen A, Bechshøft RL, Malmgaard-Clausen NM, et al. Lack of muscle fibre hypertrophy, myonuclear addition, and satellite cell pool expansion with resistance training in 83-94-year-old men and women [Internet]. *Acta Physiol Oxf Engl*. 2019; doi:10.1111/apha.13271.

Supplemental Table S1 Primary and secondary antibodies for immunohistochemistry

Staining protocol	**Primary Antibodies**
(1) Fibre CSA + Fibre Type	Laminin (rabbit anti-laminin IgG; 1:1000; Z0097; DAKO) MHC-I (mouse anti-MHC-I IgG2b; 1:200; #BA.D5; DSHB[1]) Satellite cells (mouse anti-pax7 IgG1; 1:20; DSHB[2])
(2) SCs + Fibre Type	Laminin (rabbit anti-laminin IgG; 1:1000; Z0097; DAKO) MHC-I (mouse anti-MHC-I IgG2b; 1:200; #BA.D5; DHSB) Satellite cells (mouse anti-pax7 IgG1; 1:200; DSHB)
(3) SCs + Proliferating Cells	Satellite cells (mouse anti-pax7 IgG1; 1:100; DSHB), Ki67 (rabbit anti-Ki67; 1:100; Ref: CRM325C; Biocare Medical)
Staining protocol	**Secondary Antibodies**
(1) Fibre CSA + Fibre Type	Laminin (Far-red, Alexa Fluor 680 goat anti-rabbit IgG; 1:500; A-21076, ThermoFischer) MHC-I (Red, Alexa Fluor 568 goat anti-mouse IgG2b; 1:500; A-21144, ThermoFischer) Pax7 (Green, Alexa Fluor 488 goat anti-mouse IgG1; 1:200; A-21121, ThermoFischer)
(2) SCs + Fibre Type	Laminin (Far-red, Alexa Fluor 680 goat anti-rabbit IgG; 1:500; A-21076, ThermoFischer) MHC-I (Red, Alexa Fluor 568 goat anti-mouse IgG2b; 1:500; A-21144, ThermoFischer) Pax7 (Green, Alexa Fluor 488 goat anti-mouse IgG1; 1:500; A-21121, ThermoFischer)
(3) SCs + Proliferating Cells	Pax7 (Green, Alexa Fluor 488 goat anti-mouse IgG, 1:200; A-11029, ThermoFischer) Ki67 (Red, Alexa Fluor 568 goat anti-rabbit IgG; 1:200; A-11036, ThermoFischer)
Notes	[1] BA-D5 was deposited to the DSHB by Schiaffino, S. [2] Pax7 was deposited to the DSHB by Kawakami, A.

Primary and secondary antibodies used in the three staining protocols for:
(1) Fibre CSA + fibre type
(2) Satellite cells + fibre type
(3) Satellite cells + proliferating cells
MHC = Myosin Heavy Chain
SC = Satellite cell

Supplemental Table S2 Primers for real-time PCR.

mRNA	Genbank	Sense	Antisense
MAFbx	NM_058229.3	TGTTACCCAAGGAAAGAGCAGTATGGA	ACGGAGCAGCTCTCTGGGTTATTG
MURF1	NM_032588.3	TGGGGGAGCCACCTTCCTCT	ATGTTCTCAAAGCCCTGCTCTGTCT
Myostatin	NM_005259.2	TGCTGTAACCTTCCCAGGACCA	GCTCATCACAGTCAAGACCAAAATCC
TNF-alpha	NM_000594.3	TTCCCCAGGGACCTCTCTCTAATC	GAGGGTTTGCTACAACATGGGCTAC
COL1A1	NM_000088.3	GGCAACAGCCGCTTCACCTAC	GCGGGAGGACTTGGTGGTTTT
TenascinC	NM_002160.3	CAACCATCACTGCCAAGTTCACAA	GGGGGTCGCCAGGTAAGGAG
TCF7L2	NM_001146274.1	CGGAAGGAGCGACAGCTTCAT	GTCTCTCCCGGCTGCTTGTCC
HSP70	NM_005345.5	GTGGCTGGACGCCAACACCTT	TTACACACCTGCTCCAGCTCCTTC
CD68	NM_001251.2	CAGCTTTGGATTCATGCAGGACC	CTCTGCCCCAGGGGTGCTTG
Ki67	NM_002417.4	CGGAAGAGCTGAACAGCAACGA	GCGTCTGGAGCGCAGGGATA
Myogenin	NM_002479.5	CTGCAGTCCAGAGTGGGGCAGT	CTGTAGGGTCAGCCGTGAGCAG
NCAM1	NM_000615.6	ATTGCGGTCAACCTGTGTGGAA	CCACGATGGGCTCCTTGGACT
p16(CDKN2A)	NM_000077.4	GGGGGCACCAGAGGCAGTAA	TTCTCAGAGCCTCTCTGGTTCTTTCA
IGF1-Ea	NM_000618.3	GACATGCCCAAGACCCAGAAGGA	CGGTGGCATGTCACTCTTCACTC
IGF1-Ec	NM_001111283.2	GCCCCCATCTACCAACAAGAACAC	CGGTGGCATGTCACTCTTCACTC
GAPDH	NM_002046.4	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT
RPLP0	NM_053275.3	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG