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DOI: 10.1042/CS20140447
Handle: http://hdl.handle.net/1942/17617

Neuromuscular electrical stimulation prevents muscle wasting in critically ill, comatose patients

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Running title: NMES prevents muscle atrophy during critical illness
Keywords: NMES, critical illness, muscle wasting, ICU, skeletal muscle, disuse atrophy
Clinical trial registration: NCT01521637
Word count: 3407

No funding was obtained for this study

Abstract

Fully-sedated patients, being treated in the ICU, experience substantial skeletal muscle loss. Consequently, survival rate is reduced and full recovery after awakening is compromised. Neuromuscular electrical stimulation (NMES) represents an effective method to stimulate muscle protein synthesis and alleviate muscle disuse atrophy in healthy subjects. We investigated the efficacy of twice-daily NMES to alleviate muscle loss in six fully-sedated ICU patients admitted for acute critical illness (*n*=3 males, *n*=3 females; age 63±6 y; APACHE II disease severity-score: 29±2). One leg was subjected to twice-daily NMES of the quadriceps muscle for a period of 7±1 d while the other leg acted as non-stimulated control (CON). Directly before the first and on the morning after the final NMES session, quadriceps muscle biopsies were collected from both legs to assess muscle fiber-type specific cross-sectional area (CSA). Furthermore, phosphorylation status of key proteins involved in the regulation of muscle protein synthesis was assessed, and mRNA expression of selected genes was measured. In the CON leg, type I and type II muscle fiber CSA decreased by 16±9 and 24±7%, respectively ($P<0.05$). No muscle atrophy was observed in the stimulated leg. NMES increased mTOR phosphorylation by 19% when compared to baseline ($P<0.05$), with no changes in the CON leg. Furthermore, mRNA expression of key genes involved in muscle protein breakdown either declined (FOXO1; $P<0.05$) or remained unchanged (MAFBx and MuRF1), with no differences between legs. In conclusion, NMES represents an effective and feasible interventional strategy to prevent skeletal muscle atrophy in critically ill, comatose patients.

Abstract word count: 249

Introduction

Critically ill patients suffer from extensive muscle wasting, which occurs rapidly at the onset of an ICU stay [1-3]. Aside from an increased risk of mortality [4, 5], consequences to this muscle loss include muscle weakness, prolonged mechanical ventilation, fatigue, decreases in muscle strength, impaired glucose homeostasis and delayed recovery and rehabilitation [6-9]. ENREF 5 Muscle atrophy in ICU patients exceeds that seen in normal hospitalized or bedridden persons [10, 11]. Moreover, ICU patients who are mechanically ventilated and deeply sedated are thought to be even more susceptible to muscle wasting and subsequent negative health consequences due to a complete lack of muscle contraction. Despite this, no data are currently available concerning muscle fiber atrophy in this specific ICU patient subpopulation.

Early ambulation has been proven a successful rehabilitation strategy in non-sedated ICU patients in terms of improving functional outcomes and overall prognosis [12]. However, in fully-sedated patients, early ambulation is not feasible and, as such, alternative strategies should be defined to alleviate muscle wasting. Neuromuscular electrical stimulation (NMES) is an effective means to invoke involuntary muscle contractions. Previously, NMES has been shown to attenuate the loss of muscle mass and strength experienced by non-sedated ICU patients [13] and healthy individuals subjected to limb immobilization [14]. However, the potential for NMES to rescue muscle mass in fully-sedated, comatose ICU patients has not been investigated. In the present study, we investigated our hypothesis that daily NMES attenuates skeletal muscle fiber atrophy in fully-sedated, comatose ICU patients. Fully-sedated ICU patients, expected to be sedated for a minimum of three days, were included in the present study. NMES was performed twice-daily on the quadriceps of one leg, whereas the other leg served as a sham-treated control. Prior to and immediately after the intervention, plasma samples were taken to assess any systemic changes in amino acid availability during the experiment, and muscle biopsies were taken from both legs to assess muscle fiber atrophy and myocellular characteristics. Additionally, RT-PCR and Western blotting were performed on collected muscle tissue samples to assess the potential impact of NMES on basal mRNA and protein expression levels of key genes involved in the regulation of muscle mass maintenance.

Methods

Patients

All patients admitted to the Intensive Care Unit (ICU) of Jessa Hospital, Hasselt, Belgium between March 2012 and July 2013 were assessed for eligibility for the present study (see **eFigure1**). Patients admitted to the ICU were screened by the nursing staff, and were excluded if one or more of the following exclusion criteria were met: <18 or >80 y old, not expected to undergo complete sedation, suffering from spinal cord injury, recent arterial surgery on the legs, local wounds that prohibit the application of neuromuscular electrical stimulation (NMES), chronic use of corticosteroids, intake of certain antithrombotic drugs, or the presence of an implantable cardioverter-defibrillator (ICD) and/or pacemaker. Secondly, the expected sedation time was estimated by the responsible physician and patients were excluded if this was <3 days. All patients who were excluded based on an expected short sedation time were re-evaluated after 24 h, and included if the revised expected sedation time was >3 d. Participants were accepted into the study after written informed consent was obtained from their legal representatives. The study was approved by the Medical Ethical Committee of the Jessa Hospital in accordance with the Declaration of Helsinki.

Study design

An overview of the experimental protocol is depicted in **eFigure 2**. Patients were included in the study directly after informed consent was obtained from their legal representatives, which was generally given within 2.5 d after admission to the ICU (depicted in column 'Time to inclusion' in **Table 1**). After this, patient's legs were randomly assigned as either the control (CON) or stimulated (NMES) leg, counterbalanced for left and right legs. Randomization was performed by an independent investigator, and treatment allocation was performed by using sequentially labeled envelopes which were opened after inclusion of subjects. Baseline measurements were then taken, which consisted of assessment of leg circumference (measured at different locations on the upper leg), obtaining an arterial blood sample, and obtaining a muscle biopsy from both legs. After the pre-measurements, NMES was performed twice-daily on one leg (NMES) whereas the other leg served as a control (CON). Post-measurements were performed on the final day of sedation, with a minimum study duration of 3 days and a maximum of 10 days. The study duration for each patient is depicted in **Table 1**. Post-measurements were performed prior to subjects being awake. Standard medical care was not altered, and passive mobilization was performed on both legs according to standard care procedures.

Data collection

At baseline, data on demographic and clinical characteristics of the patients were obtained, including information necessary to determine the severity of illness. These data were scored according to the Acute Physiology and Chronic Health Evaluation II (APACHE II) system with higher values indicating more severe illness and more therapeutic interventions, respectively [15].

Arterial blood samples were collected from the catheter already placed in the *arteria radialis*. Blood (10 mL) was collected into EDTA-containing tubes and immediately centrifuged at 1,000*g* for 10 min at 4°C. Aliquots of plasma were directly snap-frozen in liquid nitrogen and stored at -80°C until further analysis. Processing and storage of the samples was done by UBiLim (Universitaire Biobank Limburg, Hasselt, Belgium). Plasma amino acid concentrations

were measured using ultra-performance liquid chromatography tandem mass spectrometry as described previously [16], and results are displayed in **eTable2**.
In addition, during the pre- and post-measurements, a muscle biopsy sample was collected from each leg. After injection of local anesthesia, percutaneous needle biopsy samples were collected from *m. vastus lateralis*, approximately 15 cm above the patella using the Bergström technique [17].

Neuromuscular electrical stimulation
Neuromuscular electrical stimulation sessions were performed both in the morning (11:00 AM) and afternoon (4.30 PM). Four self-adhesive electrodes (2 mm thick, 50 x 50 mm) were placed on the distal part at the muscle belly of the *m. rectus femoris* and the *m. vastus lateralis*, and at the inguinal area of both muscles. The electrodes were connected to an Enraf-Nonius TensMed S84 stimulation device (Enraf-Nonius, Rotterdam, the Netherlands), discharging biphasic symmetric rectangular-wave pulses. The position of the electrodes was re-marked daily with a semi-permanent marker to maintain the same location of stimulation for each session. The NMES protocol was composed of a warm-up phase (5 min, 5 Hz, 250 µs), a stimulation period (30 min, 100 Hz, 400 µs, 5 s on (0.75 s rise, 3.5 s contraction, 0.75 s fall) and 10 s off), and a cooling-down phase (5 min, 5 Hz, 250 µs). The intensity of the stimulation was set to a level at which full contractions of *m. quadriceps femoris* were both visible and palpable. The intensity was raised approximately every 3 min when a full muscle contraction was no longer achieved with the current intensity. This protocol was based on our previous work showing increased rates of muscle protein synthesis after a single bout of NMES [18], and applied on the immobilized leg of healthy young adults [14]. During the NMES sessions, four electrodes and compatible cables were also applied to the control leg to standardize all procedures (representing a sham treatment).

Dietary intake
When patients were hemodynamically stable, enteral feeding was started according to routine guidelines of the ICU at Jessa Hospital as early as possible. Patients were fed Nutrison Multi Fibre (containing 420 kJ, 16 en% protein, 49 en% carbohydrates, and 35 en% fat per 100 mL). Generally, patients were fed maximally 80 mL per hour with short intervals during which nutritional supply was paused. Gastric emptying was determined by the nursing staff, and food administration was altered accordingly. Nutritional support was not modulated and was applied according to the standard medical care in this ICU.

Muscle analyses
Muscle samples were freed from any visible non-muscle tissue and separated into different sections; the first part (~30 mg) was imbedded in Tissue-Tek (Sakura Finetek, Zoeterwoude, the Netherlands), frozen on liquid nitrogen cooled isopentane and used to determine muscle fiber-type specific cross-sectional area (CSA) and satellite cell content as done previously.[19] The second part (~15 mg) was snap frozen in liquid nitrogen and used for real time-PCR analysis to determine mRNA expression of selected genes as described before,[14, 20] and compared ENREF_19 with mRNA expression of *n*=6 healthy, age- and gender-matched controls. The third part (~40 mg) was snap frozen in liquid nitrogen for Western Blot analysis to determine the total content and phosphorylation status of several key proteins of interest as described previously [18]. All muscle analyses were performed by an investigator blinded to

treatment. A detailed overview of the muscle analyses is presented in the supplemental information.

Statistics

Based on data from previous studies in healthy subjects in our laboratory [14, 21], we calculated that 8 patients would be required to detect a 8% difference in muscle fiber CSA between CON and NMES over 7 days (using an α level of 0.05 and a β level of 0.10). All data presented are expressed as means±SEM. Baseline differences between legs were compared with a paired samples t-test. Pre- and post-intervention data were analyzed using repeated measures analysis of variance (ANOVA) with time (pre vs post) and treatment (CON vs NMES) as factors. Fiber type (type I vs type II) was added as a third within-subjects factor when analyzing all muscle fiber characteristics. In case of significant interaction (*time x treatment*), paired-samples t-tests were performed to determine time effects within the CON and NMES leg separately. Alternatively, when a *time x treatment* effect was observed for muscle fiber characteristics, a 2-way ANOVA was performed for the CON and NMES leg separately, with time and treatment as factors. For the mRNA analyses, differences between patients and healthy controls were tested by means of an independent samples t-test between the mean value of the CON and NMES leg in patients and the values observed in healthy controls. Statistical analyses were performed using the SPSS version 20.0 software package (SPSS Inc., Chicago, IL, USA), with $P<0.05$ as the value for statistical significance.

Results

Patients

Between March 2012 and July 2013, 9 patients were included in the present study. Two patients awoke after <3 study days and one patient died. Therefore, the presented results represent data collected from 6 patients. Clinical characteristics of the included patients are listed in **Table 1**. Energy intake per day averaged 5.31±0.56 MJ, with a mean protein intake of 0.56±0.06 g·kg body weight^{-1}·day^{-1}.

Neuromuscular electrical stimulation

Within 5 min of the start of the actual 30 min stimulation period, a full muscle contraction was achieved. The intensity of the NMES intervention for subjects averaged 29.9 mA during the first session and was progressively increased to 32.3 mA in the final session.

Muscle fiber characteristics

Figure 1 illustrates the delta change in muscle fiber cross-sectional area (CSA) in both the NMES and CON legs throughout the study. **Table 2** details skeletal muscle fiber type specific characteristics at baseline and following 7±1 d of full sedation in both legs. In the CON leg, a significant decline of 16±9% and 24±7% was observed in type I and II muscle fiber CSA, respectively (time effect; $P<0.05$). In contrast, the NMES leg showed no atrophy in either type I or II muscle fibers (*time x treatment* interaction effect; $P<0.05$). Muscle fiber type distribution showed an overall significant *time x treatment* interaction effect (see **Table 2**; $P<0.05$), with a shift from type I towards type II fibers in the CON leg, and a shift towards more type I fibers in the NMES leg. At baseline, satellite cell content was greater in type I vs type II muscle fibers (expressed per muscle fiber, per millimeter squared, and as a percentage of total myonuclei). No differences in muscle fiber type specific myonuclear content, myonuclear domain size or satellite cell content were observed between legs or over time.

mRNA expression

Figure 2 displays the relative muscle mRNA expression of key genes involved in the regulation of muscle protein synthesis and breakdown in the CON and NMES leg before and after the intervention, as well as for a group of healthy, age- and gender-matched controls. At baseline, mRNA expression did not differ between NMES and CON legs. However, MAFBx, MuRF1, FOXO1, mTOR and P70S6K were all more highly expressed in the patients compared with healthy controls ($P<0.01$). There was a significant time effect ($P<0.05$) such that FOXO1 and P70S6K expression decreased during the period of sedation, with no differences between legs. Expression levels for all other genes did not reveal any interaction or time effects. The mRNA expression of additional genes involved in the regulation of myogenesis, oxidative metabolism, mechano-sensing and cellular amino acid transport are presented in **eFigure 3** (supplemental material).

Signaling proteins

The skeletal muscle content and phosphorylation status of key proteins involved in the regulation of muscle protein synthesis are displayed in **Figure 3**. Neither total protein content, nor phosphorylation status of Akt was affected by time or the intervention (both $P>0.05$). Whereas muscle mTOR content was unaffected by time or treatment, a significant *time x treatment*

interaction effect (P<0.05) was found for the phosphorylation status of mTOR. mTOR phosphorylation increased by as much as 19±5% in the NMES leg (P<0.05), with no changes in the CON leg (P>0.05). Muscle P70S6K total protein content decreased following the intervention in both legs (time effect, P<0.05), without changes in phosphorylation status (P>0.05).

Discussion

In the present study, we demonstrate for the first time that fully-sedated patients experience substantial type I and type II muscle fiber atrophy during a ~7 d stay in the ICU. Daily application of neuromuscular electrical stimulation (NMES) effectively prevents skeletal muscle fiber atrophy, offering an effective and feasible interventional strategy to alleviate muscle wasting in comatose ICU patients.

General admission to the ICU has been shown to cause substantial muscle wasting [22] with a decline in type I and type II muscle fiber cross-sectional area of 3% and 4% per day, respectively [2]. In keeping with this, we show a 2.8% and 4.4% decline in muscle fiber size in type I and II muscle fibers, respectively, in fully-sedated patients (i.e. no possibility of voluntary muscle contraction) during on average 7 days in the ICU (**Figure 1**). By way of comparison, muscle atrophy brought about by disuse only in healthy humans (i.e. limb immobilization) leads to a 0.5% and 0.9% per day decline in type I and II muscle fiber cross-sectional area (CSA), respectively [21]. This implies that the mechanisms responsible for muscle wasting in the ICU are not simply attributed to disuse. One possible contributing factor could be inadequate nutritional status. Sufficient dietary protein is considered a key factor in the maintenance of muscle mass [23-25], and previous research has shown that sufficient protein intake is associated with reduced mortality rates in critically ill patients [26, 27]. In the current study, patients received 0.56±0.06 g protein·kg body weight^{-1}·day^{-1}, which is below the current guidelines of 1.3-2.0 g protein·kg body weight^{-1}·day^{-1} recommended during critical illness [28, 29], and has likely contributed to the extensive level of muscle wasting. In support, plasma amino acid concentrations in our patients declined throughout the sedated state (**eTable 2**). In agreement, previous work has reported declines in circulating amino acid concentrations during critical illness [30]. Such a decline in circulating amino acid concentrations likely reduces amino acid uptake in muscle [31] and, as such, could modulate the efficacy of NMES as a means to stimulate muscle protein synthesis rates.

From a mechanistic viewpoint, disuse atrophy has been primarily attributed to declines in muscle protein synthesis rates [20, 32-34]. However, it has been suggested that in various conditions associated with rapid muscle wasting a multitude of other factors (e.g. increased inflammation, higher metabolic stress responses etc.) may stimulate muscle proteolysis, driving much of the muscle loss [35]. In line with this, we see evidence of the severely metabolically compromised condition of our patients as demonstrated by numerous clinical chemistry indictors obtained throughout the study (e.g. high white blood cell counts and C-reactive protein (CRP) concentrations; **eTable 1**). In keeping with this, molecular markers that have been used as a proxy for changes in muscle protein breakdown rate were elevated upon admission to the ICU, when compared with a group of healthy subjects (i.e. MAFBx, MuRF1 and FOXO1; **Figure 2**). The subsequent decline in the expression levels of these genes suggest a decline in muscle protein turnover during hospital stay but expression levels remained elevated when compared to healthy controls. This is not unexpected given the metabolic stress response upon ICU admission [36]. In contrast to previous work investigating the impact of NMES on an immobilized leg [14], we observed no significant differences in the expression levels of various genes between the stimulated and unstimulated leg in this comatose ICU setting. The absence of such differences may be attributed to various factors, but underline our understanding that changes in the expression and phosphorylation levels of various genes being used as a proxy for changes in muscle protein breakdown and synthesis do not necessarily represent changes in muscle protein breakdown and synthesis rates and do not necessarily translate to a net increase or decrease in

muscle mass [37]. Taken together, the present data highlight the need for immediate and effective intervention at the onset of ICU admission to stimulate muscle protein synthesis and inhibit proteolysis, thereby preventing or attenuating extensive muscle wasting. An interesting observation in the stimulated leg was that NMES reversed the decline in phosphorylation status of mTOR (**Figure 3D**), which seems to be in line with previous work showing that NMES increases muscle protein synthesis rates [18].

Daily application of NMES has been shown to prevent muscle atrophy in healthy subjects during a week of leg immobilization [14]. Moreover, clinical trials have demonstrated beneficial effects of NMES on muscle function in various bed-rested populations, including patients suffering from COPD [38, 39] and sepsis [40, 41]. The current study demonstrates, for the first time, that NMES is capable of preventing muscle wasting in fully-sedated patients during 7 days in the ICU (with a +7±12% change in mixed muscle fiber CSA in the stimulated leg compared with a -21±8% decline in mixed muscle fiber CSA in the control leg; **Figure 1)**. The prevention of muscle atrophy in these individuals can have profound clinical implications. For instance, maintaining muscle mass during critical illness has been shown to reduce mortality rates [4, 5]. Additionally, since muscle mass is vital for functional capacity [42], metabolic homeostasis [9], and immune function [43], maintaining muscle mass during an ICU stay is essential to allow proper recovery during rehabilitation. As such, preventing muscle wasting is imperative for promoting quality of life after hospital discharge and reducing the likelihood of re-hospitalization. NMES in fully-sedated patients can be easily applied by nursing staff, is relatively cheap and does not seem to cause any adverse effects on vital parameters during or after the sessions [44]. Some difficulties applying NMES in ICU patients have been reported previously and are likely due to increased skin/soft tissue impedance and/or edema [13]. Despite experiencing similar problems in the present study, all NMES sessions could be successfully performed without any adverse effects. Taken together, our data demonstrate that NMES is practical and feasible as a countermeasure for muscle wasting in clinically compromised ICU patients. Future studies should address whether these findings would translate into longer-term benefits such as increased survival rates, reduced hospitalization length of stay and/or improved rehabilitation outcomes.

Conclusion

NMES represents an effective and feasible interventional strategy to prevent skeletal muscle wasting in critically ill, comatose patients. ~~NMES may be applied effectively to offset negative consequences of muscle wasting and, as such, may increase survival and improve subsequent rehabilitation in these patients.~~

Clinical Perspectives

Fully-sedated patients experience substantial skeletal muscle loss that reduces survival rate and compromises full recovery. We investigated the efficacy of twice-daily neuromuscular electrical stimulation (NMES) to attenuate skeletal muscle loss in fully-sedated ICU patients admitted for acute critical illness. The non-stimulated leg showed substantial type I and type II muscle fiber atrophy (a 16±9 and 24±7% decline in muscle fiber cross sectional area, respectively; $P<0.05$). In contrast, no atrophy was observed in the muscle fibers collected from the stimulated leg. Both mRNA and protein expression of key proteins involved in muscle protein metabolism were assessed to understand the molecular mechanisms involved. In conclusion, NMES represents an

effective and feasible interventional strategy to prevent skeletal muscle atrophy in critically ill, comatose patients.

Author contributions

M.L. Dirks had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data. None of the authors disclose any conflicts of interest.
Study concept and design: M.L. Dirks, D. Hansen, A.van Assche, P. Dendale, and L.J.C. van Loon. *Acquisition of data:* M.L. Dirks and D. Hansen. *Analysis and interpretation of the data:* M.L. Dirks, D. Hansen, P. Dendale and L.J.C. van Loon. *Drafting of the manuscript:* M.L. Dirks. *Critical revision of the manuscript for important intellectual content:* D. Hansen, A. van Assche, P. Dendale and L.J.C. van Loon. *Study supervision:* A. van Assche, P. Dendale and L.J.C. van Loon.

Additional contributions

We gratefully acknowledge the enthusiasm and assistance of the physicians and nursing staff of the ICU in Jessa Hospital, with special thanks to Dr. P. Vranckx for his practical assistance. We would also like to thank Marika Leenders and Lex B. Verdijk for their practical support and Benjamin T. Wall for his assistance in drafting the manuscript. Furthermore, we are thankful for the assistance of dr. E. Bijnens, Department of Radiology at Jessa Hospital, and for the support and assistance provided by Biobank UbiLim at Jessa Hospital for processing and storage of the samples.

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Figure legends

Figure 1: Changes in muscle fiber cross sectional area (CSA) in the control (CON) and stimulated (NMES) leg of sedated patients, after 7±1 days of twice-daily NMES. A significant interaction effect ($P<0.05$) was observed, and a time effect in the CON leg ($P<0.05$). * Significantly ~~change~~ different from zero ($P<0.05$).

Figure 2: Skeletal muscle mRNA expression of genes of interest. Abbreviations: FOXO1, Forkhead box protein O1; MAFbx, Muscle Atrophy F-box; MuRF1, Muscle RING-finger protein-1; mTOR, mammalian target of rapamycin; P70S6K, P70S6 kinase. * Significantly different from patients at baseline ($P<0.05$). # Significantly different from pre-value ($P<0.05$).

Figure 3: Skeletal muscle protein expression of Akt, mTOR and P70S6K in the control (CON) and stimulated (NMES) leg, before (white bars) and after (black bars) 7±1 days of twice-daily NMES. Left graphs: total protein expression, right graphs: phosphorylated/total expression. Abbreviations: mTOR, mammalian target of rapamycin; P70S6K, P70S6 kinase. * Significantly different from pre-intervention values ($P<0.05$).

Figure 1:

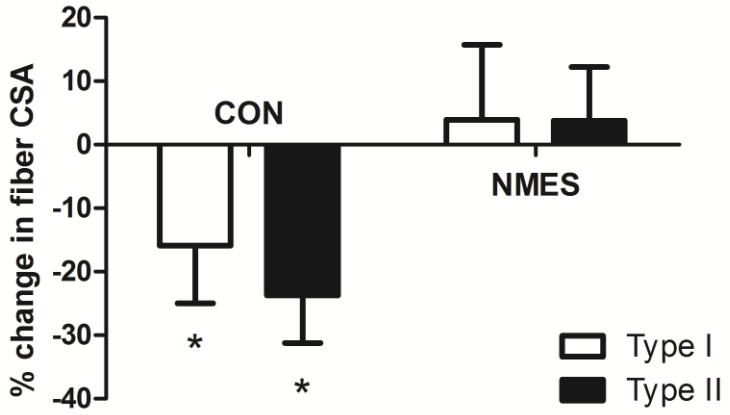

Figure 2:

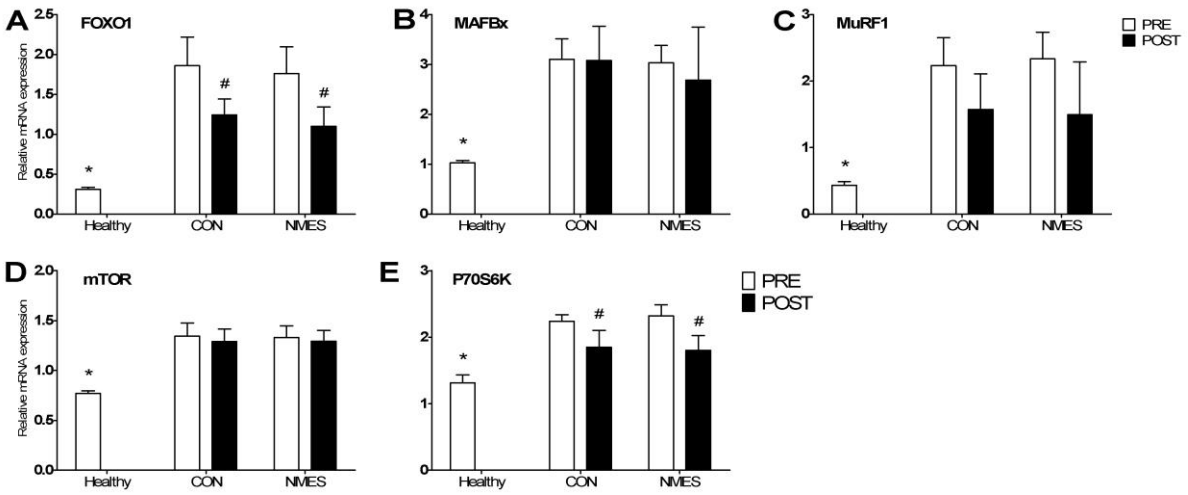

Figure 3:

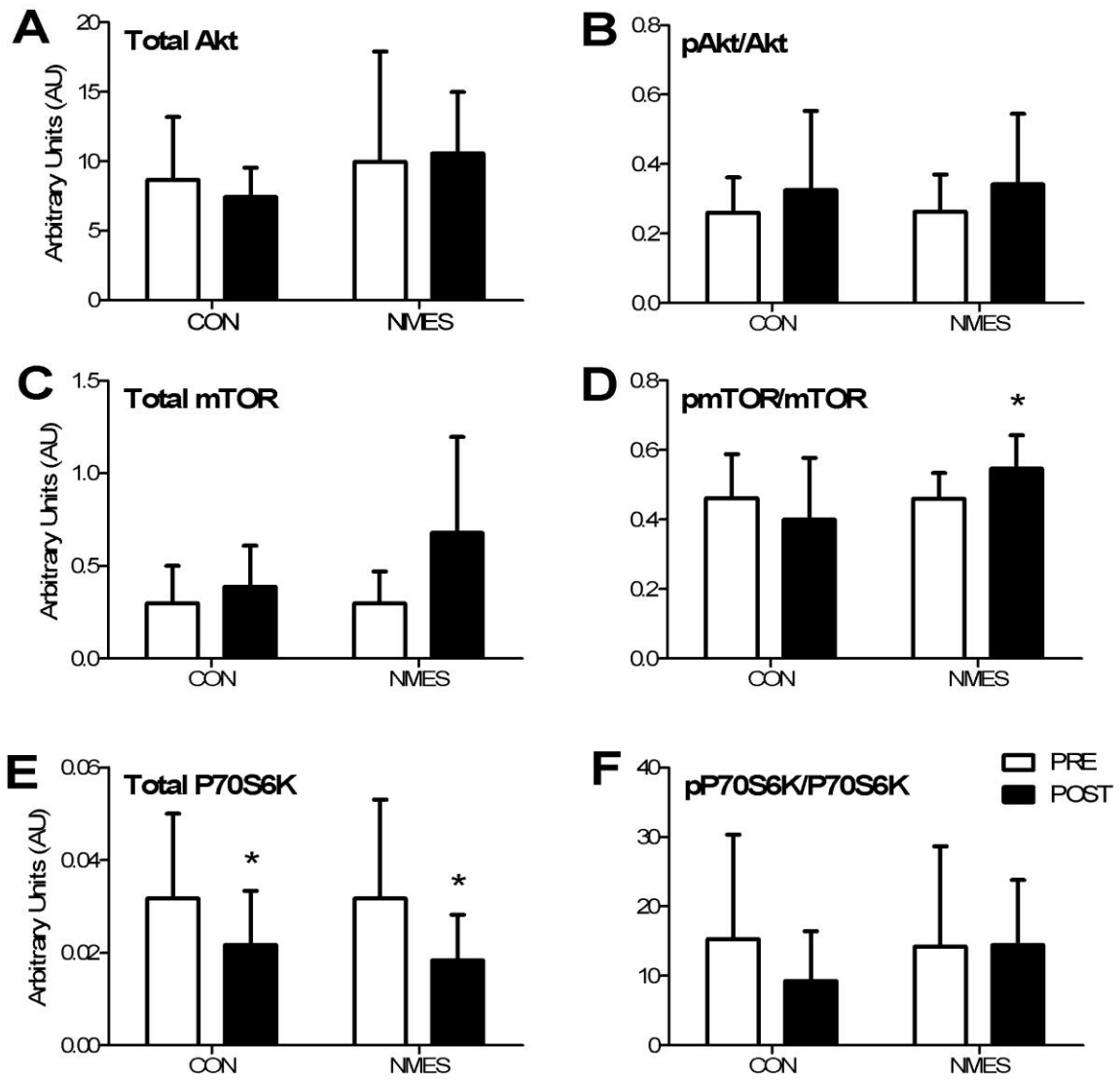
A
Total Akt
Arbitrary Units (AU)
20
15
10
5
0
CON
NMES
B
pAkt/Akt
0.8
0.6
0.4
0.2
0.0
CON
NMES
C
Total mTOR
Arbitrary Units (AU)
1.5
1.0
0.5
0.0
CON
NMES
D
pmTOR/mTOR
*
0.8
0.6
0.4
0.2
0.0
CON
NMES
E
Total P70S6K
Arbitrary Units (AU)
0.06
0.04
0.02
0.00
*
*
CON
NMES
F
pP70S6K/P70S6K
PRE
POST
40
30
20
10
0
CON
NMES

Table 1: Patients with critical illness. Fully-sedated patients were subjected to one-legged NMES for a period of 3-10 days, while the other leg served as non-stimulated control. Pre-intervention muscle measurements were performed immediately after obtaining informed content. Post-intervention measurements were performed prior to awakening after a minimum of 3 and a maximum of 10 days.

Diagnosis	Age	Sex	APACHE II at admission	Time to inclusion (d)	Days in study	Survival	Medication
Urgent CABG	78	M	32	3.5	3.5	D	Acetylcysteine, acetylsalicylic acid, alprazolam, amiodarone, amoxicillin, bisoprolol, ciprofloxacine, furosemide, haloperidol, hydrocortisone, insulin, ipratropium bromide/ fenoterol hydrobromide, isosorbide mononitrate, midazolam, milrinone, molsidomine, morphine, nadroparin, norepinephrine pantoprazole, piracetam , ramipril, spironolacton
Pneumonia	74	M	26	2.5	7.5	S	Acetylsalicylic acid, ceftazidime, ciproflocacine, erythromycin, methylprednisolone, nadroparin, pantoprazole, ranitidine
Herpetic encephalitis	39	M	25	1	9	D	Acetylcysteine, acyclovir, amoxicillin, diazepam, furosemide, HES, levetiracetam, midazolam, nadroparin, norepinephrine, paracetamol, phenytoin, propofol, thiopental, valproate
Cerebral hemorrhage	46	F	29	1	7.5	S	Aacidexam, acetylcysteine, bumetanide, ciprofloxacine, clindamycin, fluconazole, furosemide, insulin, ipratropium bromide/ fenoterol hydrobromide, lactulose, meropenem, methylprednisolone, nadroparin, norepinephrine, nystatin, pantoprazole, paracetamol, piritramide, propofol, spironolacton, valproate
Cerebral hemorrhage	78	F	29	4.5	7.5	D	Aacidexam, acetylcysteine, amoxicillin, ciprofloxacine, dobutamine hydrochloride, furosemide, insulin,

							lactulose, metoclopramide, midazolam, nebivolol, nimodipine, norepinephrine, pantoprazole, paracetamol, piritramide, pravastatin, propofol, spironolactone, timolol
Cerebral hemorrhage	65	F	35	2.5	7	D	Aacidexam, amlodipine, amoxicillin, bisoprolol, cefuroxime, ciprofloxacine, dobutamine hydrochloride, furosemide, hydrocortisone, insulin, metoclopramide, midazolam, norepinephrine, pantoprazole, paracetamol, piritramide, propofol

D = death, S = survival. Abbreviations: APACHE II = Acute Physiology and Chronic Health Evaluation II, CABG = coronary artery bypass graft, HES=hydroxyethyl starch.

Table 2: Muscle fiber characteristics

		CON		NMES	
	Fiber type	Pre	Post	Pre	Post
Muscle fiber CSA (μm²)	I	4560 ± 261	3879 ± 484 *	4414 ± 441	4512 ± 550
	II	3412 ± 530	2647 ± 512 *	3168 ± 607	3246 ± 590
% Fiber	I	53 ± 8 †	45 ± 5 †	42 ± 6 †	46 ± 6 †
	II	47 ± 8 †	55 ± 5 †	58 ± 6 †	54 ± 6 †
% Fiber area	I	59 ± 9	56 ± 8	51 ± 8	55 ± 7
	II	41 ± 9	44 ± 8	49 ± 8	45 ± 7
Nuclei per fiber	I	2.4 ± 0.1	2.5 ± 0.2	2.3 ± 0.2	2.6 ± 0.2
	II	2.1 ± 0.2 #	2.1 ± 0.2 #	1.9 ± 0.2 #	2.3 ± 0.3 #
Myonuclear domain (μm²)	I	1853 ± 69	1574 ± 183	1931 ± 110	1760 ± 142
	II	1573 ± 162 #	1255 ± 203 #	1618 ± 153 #	1452 ± 220 #
Number of SCs per fiber	I	0.083 ± 0.014	0.085 ± 0.012	0.075 ± 0.006	0.092 ± 0.006
	II	0.061 ± 0.015 #	0.049 ± 0.013 #	0.048 ± 0.008 #	0.055 ± 0.009 #
Number of SCs per mm²	I	18.8 ± 2.9	22.7 ± 2.7	17.6 ± 1.7	22.1 ± 4.1
	II	18.6 ± 2.9 #	17.8 ± 2.0 #	17.0 ± 3.9 #	19.8 ± 4.6 #
SCs/myonuclei (%)	I	3.4 ± 0.5	3.5 ± 0.4	3.4 ± 0.4	3.7 ± 0.4
	II	2.7 ± 0.4 #	2.3 ± 0.5 #	2.5 ± 0.3 #	2.5 ± 0.3 #

Data represent means±SEM. Abbreviations: SC, satellite cell; SCs/myonuclei (%), the number of SCs as a percentage of the total number of myonuclei (i.e. number of myonuclei + number of SCs). * Significantly different from pre-intervention value ($P<0.05$). # Significantly different from type I fiber value ($P<0.05$). † Significant treatment*time*fiber type interaction effect ($P<0.05$)

Supplemental material

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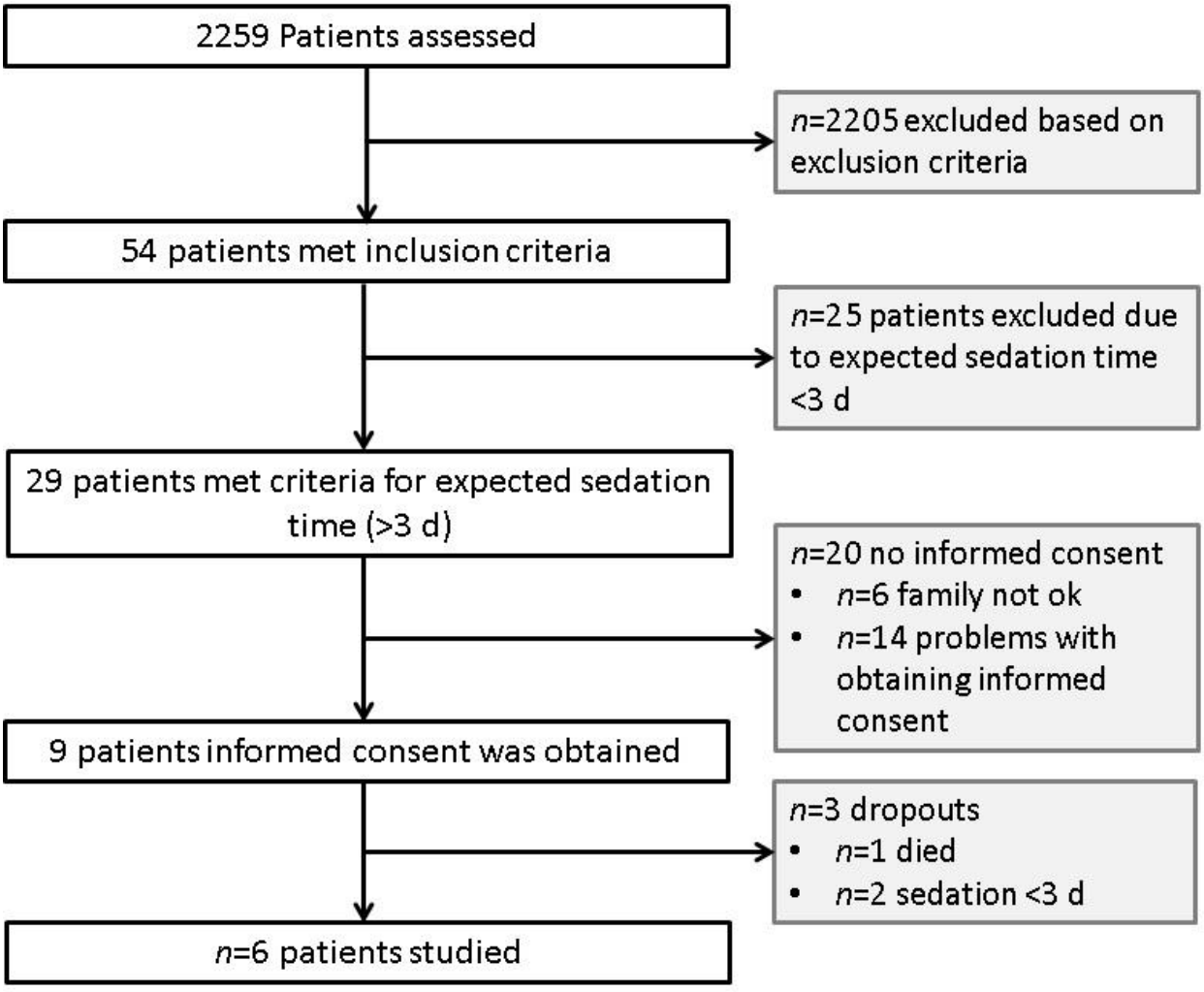
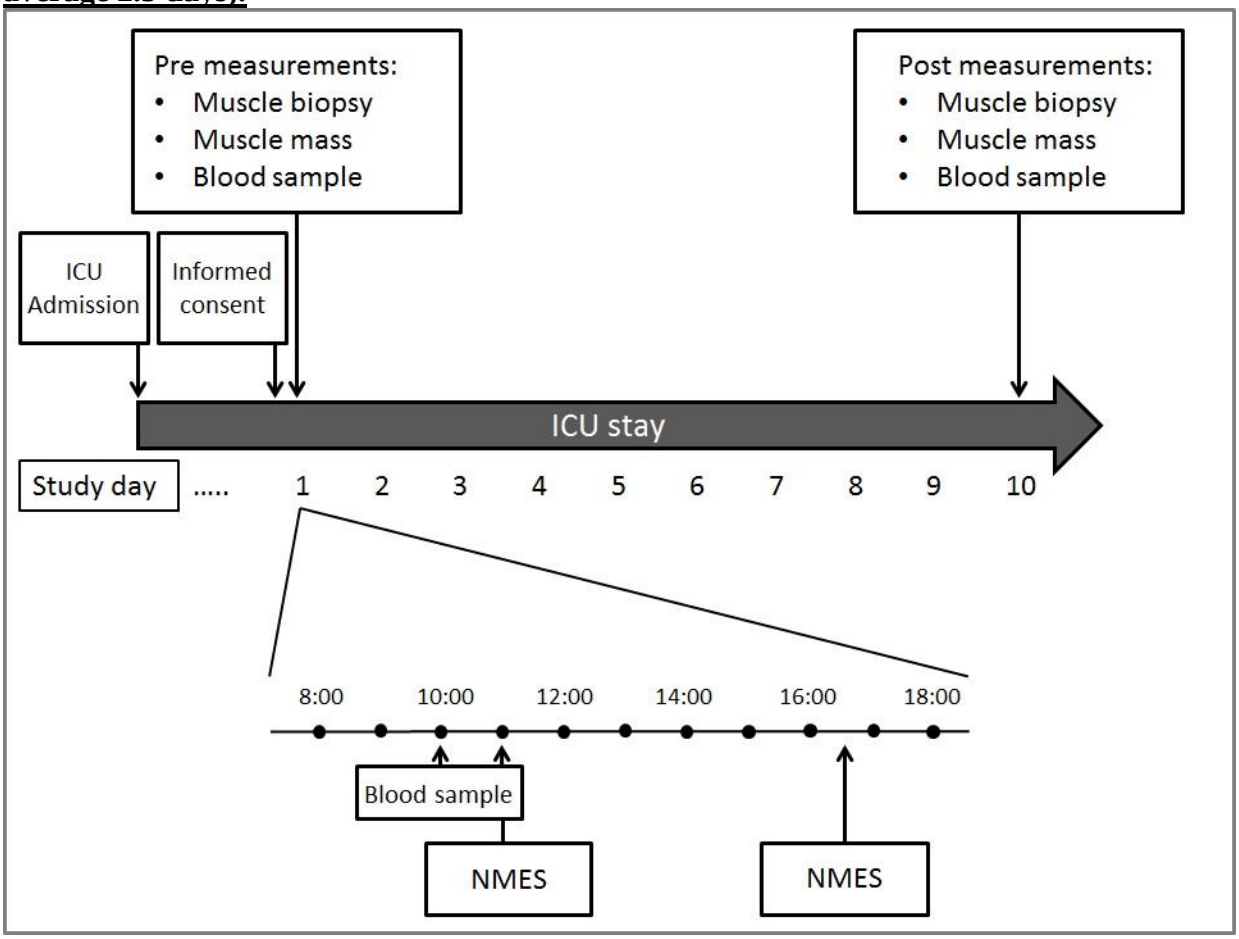
eMethods

eFigure 1: Patient flow



eFigure 2: Overview of the experimental protocol. Upper part: Overview of the study, in which fully-sedated patients were subjected to minimally 3 days and maximally 10 days of NMES. Post-intervention measurements were performed prior to patient awakening. Lower part: detailed overview of each study day. '…' represents the duration between ICU admission and obtaining informed consent from the patient's legal representatives (i.e. on average 2.5 days).



Leg circumference measurements

In the morning of day 1, circumference of the legs was measured using a tape measure at 5, 10 and 20 cm proximal to the top of the patella. The positions of leg circumference measurements were marked with semi-permanent ink to ensure the exact same measuring position during the post-measurements.

Muscle analysis

The part of the muscle sample that was mounted and frozen in Tissue-Tek (Sakura Finetek, Zoeterwoude, the Netherlands) was cut into 5µm thick cryosections using a cryostat at -20°C. Pre- and post-intervention samples of the NMES and CON leg of each subject were mounted together on uncoated, pre-cleaned glass slides, while care was taken to properly align the samples for cross-sectional fiber analyses. Staining were performed to measure muscle fiber typing (FT) and satellite cell (SC) content. To do this, slides were incubated with primary

antibodies directed against myosin heavy chain (MHC)-I (A4.840, dilution 1:25; Developmental Studies Hybridoma Bank, Iowa City, IA), laminin (polyclonal rabbit anti-laminin, dilution 1:50; Sigma, Zwijndrecht, the Netherlands) and Pax7 (neat; cell supernatant from cells obtained from the Developmental Studies Hybridoma Bank, Iowa City, IA, USA). Pax7 has been extensively used in the determination of SC content in human skeletal muscle by us [1] and others [2]. After washing, the appropriate secondary antibodies were applied: goat anti-rabbit IgG AlexaFluor647, goat anti-mouse IgM AlexaFluor555, and Streptavidin Alexa 488 (dilution 1:400, 1:500, and 1:200, respectively; Molecular Probes, Invitrogen, Breda, the Netherlands). Myonuclei were stained with 4,6-diamidino-2-phenylindole (DAPI, 0.238 µM; Molecular Probes). Both primary and secondary antibodies were diluted in 0.1% Bovine Serum Albumin (BSA) in 0.1% Tween-phosphate-buffered saline (PBS). All incubation steps were performed at room temperature. Staining of the muscle tissue was done as follows. After fixation in acetone for 5 min, slides were air dried and incubated with 3% BSA in 0.1% Tween-PBS for 30 min. Slides were washed in PBS for 5 min, and incubated with Pax7 for 2 h. Thereafter, slides were washed (standard washing protocol: 5 min 0.1% Tween-PBS, 2x5 min PBS) and incubated with goat anti-mouse Biotin (dilution 1:133, Vector Laboratories, Inc., Burlingame, CA, USA) for 60 min, washed again, and incubated with Streptavidin for 60 min. After washing, primary antibodies against laminin and MHC-I were applied for 45 min. Slides were then washed and incubated with the appropriate secondary antibodies, diluted together with DAPI. After a last washing step, slides were mounted with cover glasses Mowiol (Calbiochem, Amsterdam, the Netherlands). Staining procedures resulted in images with nuclei stained in blue, Pax7 in green, MHC-I in red, and laminin in far-red. Images were visualized and automatically captured at 10x magnification with a fluorescent microscope equipped with an automatic stage (IX81 motorized inverted microscope, Olympus, Hamburg, Germany) and EXi Aqua CCD camera (QImaging, Surrey, BC, Canada). Image acquisition was done by Micromanager 1.4 software [3], and images were analyzed with Image J software package (version 1.46r, National Institute of Health [4]). Recording and analyzing of the images was done by an investigator blinded to subject coding. As a measure of fiber circularity, form factors were calculated by using the following formula: $(4\pi \cdot CSA)/(perimeter)^2$. No differences in fiber circularity were observed between legs or between pre- and post-intervention samples. Mean numbers of 257±33 and 238±23 muscle fibers were analyzed in the pre- and post-intervention samples, respectively.

The part of the muscle sample that was frozen in liquid nitrogen was used to determine mRNA and protein expression of several genes of interest. Frozen muscle (10-20 mg) was used for mRNA isolation using Tri Reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's protocol. Quantification of total RNA was carried out spectrophotometrically at 260 nm using the NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Madison, WI, USA), and RNA purity was determined as the ratio of readings at 260/280 nm. Thereafter, first strand cDNA was synthesized from 1 µg RNA sample using random primers (Promega, Madison, WI, USA) and PowerScript Reverse Transcriptase (Applied Biosystems, Foster City, CA, USA). Taqman PCR was carried out using an ABI Prism 7000 sequence detector (Applied Biosystems, USA), with 2 µL of cDNA, 18 $\mu l \cdot L^{-1}$ of each primer, 5 $\mu l \cdot L^{-1}$ probe, and Universal Taqman 2 × PCR mastermix (Eurogentec S.A., Seraing, Belgium) in a 25 µL final volume. Each sample was run in duplicate, in duplex reactions. A separate standard curve was included for each gene, with serial dilutions of cDNA synthesized in parallel with the study samples. 18S was selected as a housekeeping gene to be used as an internal control, as we have used it previously and have shown that it does not change with muscle disuse [5, 6], ENREF_14 ENREF_42 ENREF_15

i.e. mean C*t* values did not change over time in either leg (data not shown). Taqman primer/probe sets (Applied Biosystems) were obtained for the following genes of interest: mammalian target of rapamycin (mTOR), P70S6 kinase (P70S6K), myogenic factor 4 (myogenin), MyoD, myostatin, Atrogin-1/Muscle Atrophy F-box (MAFbx), Muscle RING-finger protein-1 (MuRF1), Forkhead box protein O1 (FOXO1), Focal Adhesion Kinase (FAK), large neutral amino acid transporter (LAT1) and Proton-coupled amino acid transporter 1 (PAT1). All genes of interest were labeled with the fluorescent reporter FAM. Thermal cycling was performed using the following conditions: 2 min at 50ºC, 10 min at 95ºC, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. C*t* values of the genes of interest were normalized to C*t* values of 18S, and these values were calculated as relative expression against the standard curve. Muscle samples from age- and sex-matched healthy controls were analyzed to compare pre-intervention values of the patients in the study to healthy volunteers.

Muscle homogenates for Western blotting analysis were made using ~40 mg of muscle tissue in 7x volumes Tris buffer (20 mM Tris-HCL, 5 mM EDTA. 10 mM Na-pyrosphospate, 100 mM NaF, 2 mM Na3VO4, 1% Nonident P-40; pH 7.4) supplemented with the following protease and phosphatase inhibitors: Aprotinin 10 µg/mL, Leupeptin 10 µg/mL, Benzamidin 3 mM and PMSF 1 mM. Muscle samples were centrifuged for 10 min at 10,000 g (4ºC), where after sample buffer (final concentration: 60 mM Tris, 5% glycerol, 20 mg/mL SDS, 0.1mM DTT, 20 µg/mL bromophenolblue) was added to the supernatant. The solution was then boiled for 5 min at 100ºC, after which the samples were put on ice. Directly before the commencement of the analyses, the extraction sample was warmed to 50 ºC and centrifuged for 1 min at 1,000 g at room temperature. Each lane on the gel was loaded with a total of 50 µg muscle sample. Protein samples (for (p)Akt, (p)P70S6K and α-actin quantification) were run on a Criterion 'any kD' gel (Biorad Order No. 567-1124) for 10 min at 50 V (constant voltage) and ±90 min at 150 V (constant voltage) and transferred onto a Trans-blot Turbo 0.2 µm nitrocellulose membrane (Biorad Order No. 170-4159) in 7 min at 2.5A and 25V. For (p)mTOR protein a Tris-acetate gel was used (Biorad Order nr. 345-0129), and the gel was transferred onto a Trans-blot Turbo 0.2 µm nitrocellulose membrane (Biorad Order No. 170-4159) in 10 min at 1.3A and 25V. Specific proteins were detected by overnight incubation at 4ºC on a shaker with specific antibodies in 50% PBS/Odyssey blocking buffer (Li-Cor Biosciences Part No. 927-40000) after blocking for 60 min at RT in 50% PBS/Odyssey blocking buffer. Antibodies that were used in this study were anti-Akt (60 kD; dilution 1:1000, #9272 Cell Signaling) and anti-phospho-Akt (Ser473; 60 kD, dilution 1:1000, #9271 Cell Signalling), anti-mTOR (289 kD; dilution 1:1000, #2972 Cell Signalling) and anti-phospho-mTOR (Ser2448; 289 kD, dilution 1:1000, #2971 Cell Signalling), anti-P70S6K (70 kD; dilution 1:1000, #9202 Cell Signalling), anti-phospho P70S6K (Thr389; 70kD, dilution 1:1000, #9206 Cell Signalling) and anti α-actin (42 kD; dilution 1:100.000, mouse monoclonal IgM, Sigma A2172). After incubation, membranes were washed 3 x 10 min in 0.1% PBS-Tween and 1 x 10 min with PBS. Samples were incubated for 1 h at room temperature with the following secondary antibodies: donkey anti-rabbit IRDYE 680 (Li-Cor, Cat. No. 926-32223, dilution 1:10000) and donkey anti-mouse IRDYE 800CW (Li-Cor, Cat. No. 926-32212, dilution 1:10000) dissolved in 50% PBS Odyssey blocking buffer. After the last washing step (3 x 5 min in 0.1% Tween20-PBS and 1 x 10 min with PBS), protein quantification was performed by scanning on an Odyssey Infrared Imaging System (LI-COR Biotechnology, Lincoln, NE, USA).

eResults

Laboratory results

eTable1: Average laboratory values during the study period

	Value	Reference value
White blood cell count (*10^9/L)	15.9 ± 2.5	4.5 – 11.0
Lymphocytes (*10^9/L)	0.8 ± 0.1	1.01 – 3.38
Monocytes (*10^9/L)	0.90 ± 0.30	< 0.82
CRP (mg/dL)	9.1 ± 2.3	< 0.5
Ureum (mg/dL)	74.6 ± 10.9	17-43
Creatinine (mg/dL)	0.9 ± 0.1	0.51 – 0.95
Total plasma protein (g/dL)	5.7 ± 0.2	6.6 – 8.3
Albumin (g/dL)	2.8 ± 0.1	3.5 – 5.2

CRP = C-reactive protein

Plasma amino acid concentrations

Plasma concentrations of the measured amino acids are displayed in **eTable2**. Following the intervention, significant decreases in the concentrations of alanine, histidine and phenylalanine were observed (all $P<0.05$), whereas for leucine a trend for a decline was seen ($P=0.065$).

eTable 2: Plasma AA concentrations

	Pre	Post
α-aminobutyric acid	47 ± 10	29 ± 4
Alanine	369 ± 50	241 ± 31 *
Arginine	66 ± 16	47 ± 8
Asparagine	63 ± 10	44 ± 5
Aspartic acid	4 ± 1	6 ± 2
Citrulline	18 ± 3	23 ± 4
Cysteine	40 ± 11	48 ± 9
Glutamic acid	46 ± 15	73 ± 20
Glutamine	470 ± 59	430 ± 70
Glycine	263 ± 67	189 ± 24
Histidine	81 ± 4	55 ± 6 *
Isoleucine	77 ± 6	60 ± 7
Leucine	158 ± 9	111 ± 14
Lysine	218 ± 38	145 ± 19
Methionine	40 ± 6	28 ± 5
Ornithine	90 ± 18	78 ± 11
Phenylalanine	99 ± 10	71 ± 8 *
Proline	161 ± 21	158 33
Serine	85 ± 14	69 ± 9
Taurine	42 ± 12	45 ± 8
Threonine	139 ± 28	91 ± 16
Tryptophan	34 ± 7	31 ± 6
Tyrosine	77 ± 13	61 ± 8
Valine	283 ± 22	222 ± 24

Data are presented as means ± SEM.* Significantly different from pre-value (P<0.05).
All values are presented as μmol/L.

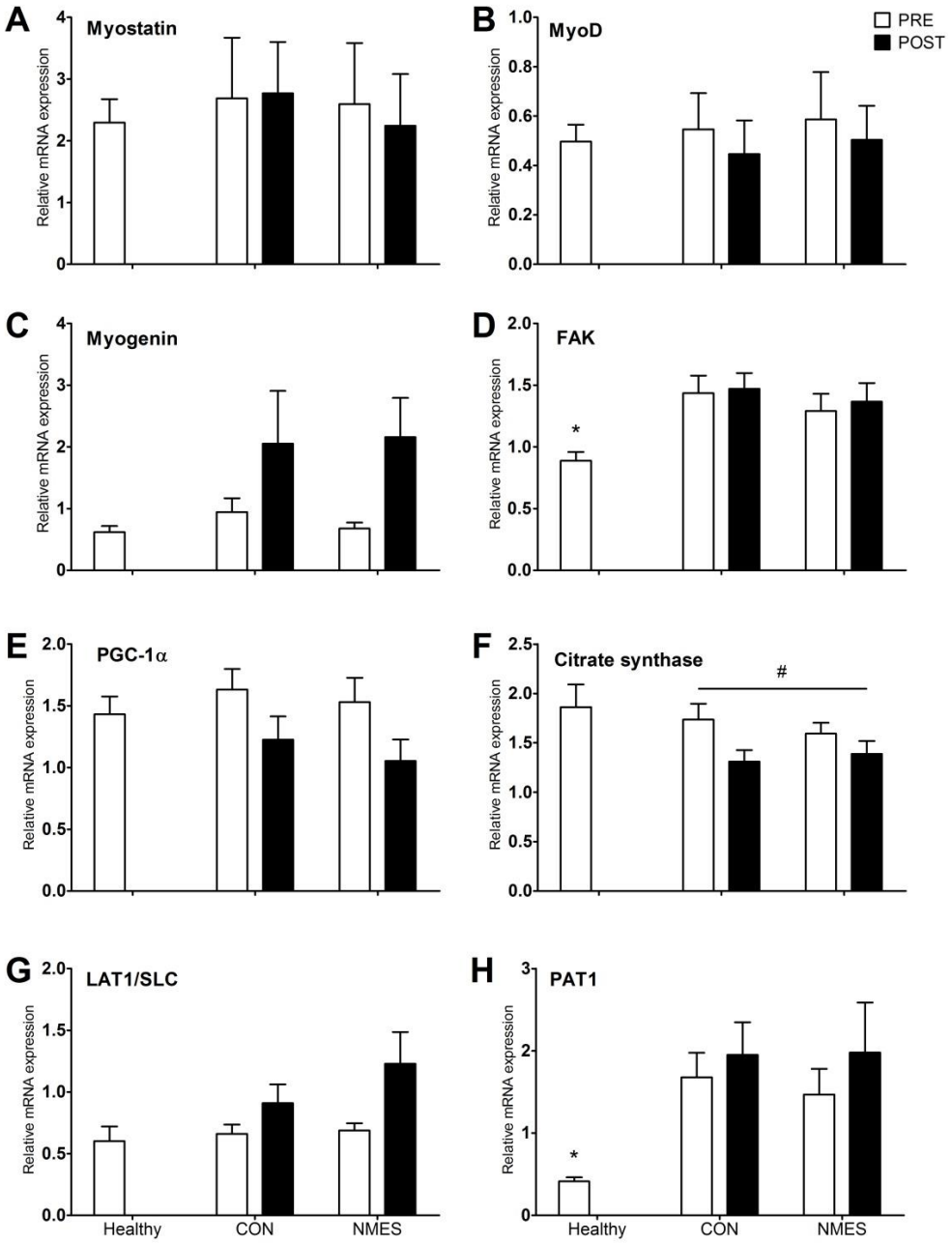
Leg circumference

No baseline differences in leg circumference between NMES and CON legs were observed, and no effect of time or treatment occurred (both interaction and time effect $P>0.05$).

mRNA analysis

For myostatin (**eFigure 3A**), myoD (**eFigure 3B**), and myogenin (**eFigure 3C**), no differences were observed when compared with healthy controls and no interaction effect was found for the CON and NMES leg (all $P>0.05$). FAK (**eFigure 3D**) was higher in patients than in healthy controls ($P<0.05$), but was not changed over time or between legs ($P>0.05$). For both PGC-1α (**eFigure 3E**) and citrate synthase (**eFigure 3F**) no differences between healthy controls and patients were observed (both $P<0.05$). Although not significantly ($P>0.05$), PGC-1α tended to decrease over time in both legs. Citrate synthase showed a significant time*treatment interaction ($P<0.05$); when both legs were tested separately with a paired-samples t-test a trend for a decline in the CON leg ($P=0.059$) was observed, while in the NMES leg no decrease was observed ($P>0.05$). The amino acid transporters LAT1 (**eFigure 3G**) and PAT1 (**eFigure 3H**) were not different (LAT1) or significantly higher (PAT1; $P<0.01$) in patients compared with healthy controls, whereas over time no changes were observed in either gene (both $P>0.05$).

eFigure 3: Skeletal muscle mRNA expression of genes of interest in the CON and NMES leg of fully-sedated ICU patients (right part of the figure) versus healthy controls (left column), before (white bars) and after (black bars) 7±1 days of twice-daily NMES. Abbreviations: FAK, Focal Adhesion Kinase; LAT1, Large Neutral Amino Acid Transporter 1; PAT1, Proton-coupled amino acid transporter 1; PGC-1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha. * Significantly different from the mean pre-intervention value of the CON and NMES legs. # Significant interaction effect (P<0.05).



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