Online Data Supplement

Muscle wasting and function after muscle activation and early protocol-based physiotherapy: an explorative trial

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Study Design

The trial is a prospective randomised controlled interventional trial (ISRCTN19392591). Enrolment of patients took place in two intensive care units within the Department of Anesthesiology and Operative Intensive Care Medicine (CCM, CVK) at Charité - Universitätsmedizin Berlin, Germany. The Charité institutional review board granted ethical approval (Charité EA 2/041/10).

Participants

Inclusion criteria:

- Mechanical ventilation
- SOFA ≥ 9 within the first 72 hours after ICU admission

Exclusion criteria:

- age < 18 years
- insulin-dependent diabetes mellitus
- body-mass-index > 35 kg/m^2
- preexisting neuromuscular disease
- moribund health status
- participation in another clinical trial
- prior ICU treatment or mechanical ventilation for more than 72 hours before inclusion
- pregnancy
- not ambulating before admission

Enrolment process

Patients were screened daily on ward rounds through participating ICUs by study physicians for eligibility to be enrolled into the trial. In case of fulfillment of all inclusion criteria without applicability of an exclusion criterion the legal proxy was approached for enrolment into the trial.

Muscle biopsy samples (n = 6) from patients undergoing elective orthopedic surgery but who were otherwise healthy were included as references to determine baseline values. Similarly, plasma sampls from healthy volunteers were included to determine baseline values for myostatin plasma concentrations (n = 91).

Randomisation

Primary randomisation with a 1:2 ratio into two groups of 30 [control group and intervention group] was done with sealed opaque envelopes. Sequential allocation of patients to first NMES + WBV, second NMES and, third

WBV within the intervention group was done for further subrandomisation. Study staff was blinded during the assessment of all outcome parameters and had no influence on treatment decisions.

Procedures

General ICU treatment adhered to published standard operating procedures [1-3].

Protocols for performed interventions

Protocol-based physiotherapy

Protocol-based physiotherapy was performed twice daily for 25/35 minutes by a trained and dedicated study physiotherapist seven days a week. Further muscle activating measures were performed daily by trained study staff as described below. Every morning the mobilisation goal was defined by a multiprofessional case conference considering patients ability to participate in terms of consciousness, haemodynamic stability and respiratory stability as outlined in Supplement Table S1.

1 **Table S1** Physiotherapy protocol

Level of mobilisation	**Level 1**	**Level 2**	**Level 3**	**Level 4**	**Level 5**
Description of the level of mobilisation according to patient's status	RASS: -5 • haemodynamically unstable • respiratorically unstable • ICP not compensable • "minimal-handling"	RASS: -5 to -4 • haemodynamically stable • respiratorically stable • ICP stable	RASS: -3 to -2 • haemodynamically stable • respiratorically stable • ICP stable • active participation	RASS: ≥-2 • haemodynamically stable • respiratorically stable • ICP stable • active participation	RASS: ≥-1 • no intensive care monitoring necessary • transfer to general ward planned
Mobilisation	Ø	• passive mobilisation of upper and lower extremities	• passive mobilisation • assistive mobilisation • active mobilisation	• passive mobilisation • assistive mobilisation • active mobilisation • activities of daily living	• passive mobilisation • assistive mobilisation • active mobilisation • activities of daily living • intensified therapy
Respiratory therapy	Ø	• as medically indicated • pneumonia and atelectasis prophylaxis	• as medically indicated • pneumonia and atelectasis prophylaxis	as medically indicated • pneumonia and atelectasis prophylaxis	as medically indicated • pneumonia and atelectasis prophylaxis
Active transfer	Ø	Ø	• increasing mobility within the bed	• supine → lateral → sitting → standing (assistive	•• supine → lateral → sitting → standing (assistive

(Mobilisation with participation of the patient)				devices are permitted)	devices are permitted)
Mobility training	Ø	Ø	Ø	yes	yes
Passive transfer (Mobilisation without participation of the patient)	Ø	• sitting in the Thekla®* • standing in the Thekla® *	• sitting in the Thekla®* • standing in the Thekla®*	If necessary • sitting in the Thekla®* • standing in the Thekla®*	If necessary • sitting in the Thekla®* • standing in the Thekla®*
Positioning therapy	• Prophylaxis • specific positioning	• Prophylaxis • specific positioning	• Prophylaxis • specific positioning	• Prophylaxis • specific positioning	• Prophylaxis as required • specific positioning
Frequency	NA	• 2x daily	• 2x daily	• 2x daily	• 2x daily
Duration per session	NA	• 25-35 minutes	25-35 minutes	25-35 minutes	25-35 minutes

**Thekla® refers to a chair produced by Hanse-Medizintechnik, which was developed as an assistive device that enables passively transferring an unconscious patient from supine into upright standing position.*

Protocol for neuromuscular electrical stimulation

Neuromuscular electrical stimulation (NMES) was performed bilaterally on 8 different muscle groups (M. tibialis anterior, M. triceps surae, M. vastus lateralis, posterior thigh, M. biceps brachii, M. triceps brachii, wrist extensors, wrist flexors) daily for 20 minutes starting on the day of enrolment (MUSKELaktiv 2-Kanal, schwa-medico®, Germany; Physiomed-Expert-2-Kanal, Physiomed®, Germany). Electrical impulses of 350 µs at 50 Hs with a ramp of 1 second and an on-time of 6/10 seconds as well as an off-time of 10/15 seconds. Electrical current was increased to maximal 70 mA until visible or palpable muscle contraction in unconscious respectively contraction or discomfort in awake patients occurred. If no contraction could be observed NMES was performed with 40 mA.

Protocol for Whole-body vibration

Whole-body vibration was performed daily for 20 cycles (alternating stimulation, 26 Hs, amplitude 15 mm) with one minute stimulation and a one minute break using the Galileo® (Novotec®, Germany) instrument. To assure a complete patient-instrument coupling haemodynamically stable patients were brought into an almost upright

position (80-90° with 90° meaning upper body was perpendicular to the floor) with the use of a Thekla® (Hanse-Medizintechnik, Germany) while lightly flexing their knees (0/10°). Haemodynamically unstable patients received whole-body vibration within the bed with head raised and legs lowered up to 30°. Furthermore, to ensure patient-instrument coupling knees and hips were flexed lightly (knees: 0/10° and hip: 10/30°).

Protocol for first adequate awakening and MRC

Screening for adequate awakening was performed daily by study physicians. In order to be classified as adequately awake a patient had to have a Richmond Agitation and Sedation Score between -1 and +1 as well as an adequate response to three out of the five following verbal commands: "Open/close your eyes," "Look at me," "Open your mouth and put out your tongue," "Nod your head," and "Raise your eyebrows when I have counted up to 5." on two consecutive days as previously published by DeJonghe et al.[4]. Medical Research Council score was assessed by trained study staff on a 6 point scale (0 no visible or palpable contraction; 1 visible or palpable contraction without limb movement; 2 movement without gravity; 3 movement against gravity; 4 movement against resistance; 5 full force) in 8 different muscle groups bilaterally (wrist extension, wrist flexion, elbow extension, elbow flexion, should aberduction, hip flexion, knee extension, ankle extension, ankle flexion). The sum score was divided by the number of muscle examined.

Protocols for performed analyses

Protocol for muscle biopsy and histological analyses

Muscle biopsy specimen were obtained 15 days after onset of critical illness respectively the closest day to this date in case of any circumstances not allowing muscle biopsy on the predefined date (Ethical approval: Charité EA 2/041/10). Afterwards Lidocain was administered as a local anaesthetic to the incision site located in the distal third of the *Vastus lateralis* muscle. A surgical incision through skin and fascia was done to expose muscle tissue and retrieve the muscle biopsy specimen. If necessary bleeding was stopped and the wound was closed as well as dressed. We obtained biopsy specimens from 11 patients in the control group and 26 patients in the intervention group. Furthermore 6 control biopsy specimens were obtained from volunteers undergoing elective orthopaedic surgery.

For gene expression and protein analyses biopsy specimens were directly snap frosen in liquid nitrogen and for immunohistochemistry and metachromatic ATPase staining they were mounted and frozen under cryoprotection. Specimens were stored at -80°C.

Biopsy specimens for histological analyses (Haematoxylin & Eosin or Gomori trichrome) were fixed in 3.7% paraformaldehyde, embedded into paraffin and sectioned using a cryotome (Leica CM3050 S) into 10 µm thick sections. Haematoxylin and Eosin and Gomori trichrome staining was performed as recently published [5-9]. For

metachromatic ATPase staining muscle biopsy specimens were embedded in tissue-freezing medium (Triangle Biomedical Sciences, Durham, NC) supplemented with gum tragacanth (Sigma, St. Louis, MO). Subsequently the samples were immediately frozen in liquid nitrogen-cooled isopentane (2-Methylbutan; Fa. Carl Roth) and stored afterwards in liquid nitrogen until sectioning. Mounted tissue samples were sectioned with a cryotome (Leica CM3050 S, 10 µm) for metachromatic ATPase staining which was performed as previously published [5, 8, 10]. Myocyte cross sectional area was determined on pictures of histological sections of in average 115.5 (IQR: 106 - 136) myofibers per patient from 6 control patients, 19 patients in the standard physiotherapy group, 11 patients in the control group and 25 patients in the intervention group with Image J (Version 1.47v).

GLUT4 immunohistochemical stains were performed with a rabbit anti-GLUT4 antiserum (1154p) provided by Hoffmann-La Roche.

Protocol for quantification of gene expression

TRIzol® Reagent (Invitrogen) was used to extract total RNA from muscle biopsy specimens according to the manufacturer's protocol and as recently published [[5, 6, 9]. Reverse-transcription of 1 µg RNA into cDNA was performed by using the SuperScript® First-Strand Synthesis System (Invitrogen) according to the manufacturers protocol and as recently published [5, 6, 8, 9]. TaqMan® Universal PCR Mastermix (Applied Biosystems) was used for real-time polymerase chain reaction (RT-PCR) together with commercially available primer and probe sets (Applied Biosystems) (see Table S2). Step-One™ Plus thermocycler (Applied Biosystems) was used for all PCR reactions. All experiments were conducted as manufacturer's instructions stated. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene expression was used to normalise gene expression, in order to correct for a variance in mRNA extraction and cDNA synthesis efficiency between samples. For normalisation values of volunteers undergoing elective orthopaedic surgery were set as one and patient values were expressed as fold change [5, 8, 10].

Table S2 Specifications of gene expression assays from Applied Biosystems

Gene name	**Catalogue number**
MYH1	Hs00428600_m1
MYH2	Hs00430042_m1
MYH4	Hs00757977_m1
TRIM63	Hs00822397_m1
TRIM62	Hs00217089_m1
FBXO32	Hs00369714_m1

MYH indicates myosin heavy chain; TRIM, tripartite motif containing protein and FBXO, F-box containing protein.

Protocol for protein analyses

All protein analyses were performed as previously published [5, 8, 10]. Skeletal muscle biopsy specimen were homogenised (30s, 2000 rpm) in ice-cold extraction buffer 1:3 wt/vol (10 mM Tris HCl, pH 7.5, 140 mM NaCl, 1 mM EDTA, 25% glycerol, 0.5% sodium dodecyl sulfate (SDS), 0.5% Nonident P-40) supplemented with 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 100 ng/ml protease inhibitor cocktail (Roche). Clearing was done through centrifugation (4°C, 10 min, 14000 rpm). The supernatant was assayed for protein concentration using Bio-Rad Protein Assay and stored at -80°C until analyses. SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins according to their molecular weight. Afterwards proteins were blotted onto nitrocellulose or PVDF membranes (Amersham Pharmacia Biotech). Primary and secondary antibodies used are shown in Table S3. Visualisation was performed by enhanced chemiluminescence (ECL) detection reagent (Amersham Pharmacia Biotech).

Table S3 Specification for antibodies used for Western Blots

Antibody	Clone	Manufacturer	Concentration	Membrane
anti-total Myosin heavy chain	MF20	Sigma	1:3000	nitrocellulose
anti-fast Myosin heavy chain	MY32	Sigma	1:3000	nitrocellulose
anti-slow Myosin heavy chain	NOQ7	Sigma	1:3000	nitrocellulose
anti-MuRF1		R&D	1:200	PVDF
anti-Atrogin1		Abcam	1:500	PVDF
anti-mouse IgG HRP		CellSignaling	1:3000	
anti-goat IgG HRP		Abcam	1:3000	

Plasma analysis

Myostatin plasma concentration were analysed with R&D Systems GDF-8/Myostatin Quantikine ELISA Kit (Catalogue number DGF80)

Statistics

Counts and percentages are used to present categorical variables and median and interquartile range to present metric variables. Due to small group size non-normal distribution was assumed. Statistic test were selected accordingly. Specifically, non-parametric tests for metric variables and differences between groups. Kruskal-

Wallis and Mann-Whitney U tests were used for independent samples and Wilcoxon signed-rank test test for dependent samples. Chi-Square test was used for group differences and categorical variables. $P < 0.05$ was accepted as significant. Myocyte cross sectional area shift was analysed through ANOVA and validated through Welch- and Brown-Forsythe test in case of inhomogenous variance tested by the Levene's Test. Statistical analyses were performed with SPSS IBM (version 25), and graphics were created with GraphPad Prism (version 7.0) and Sigma Plot (version 12.0).

Supplement results:

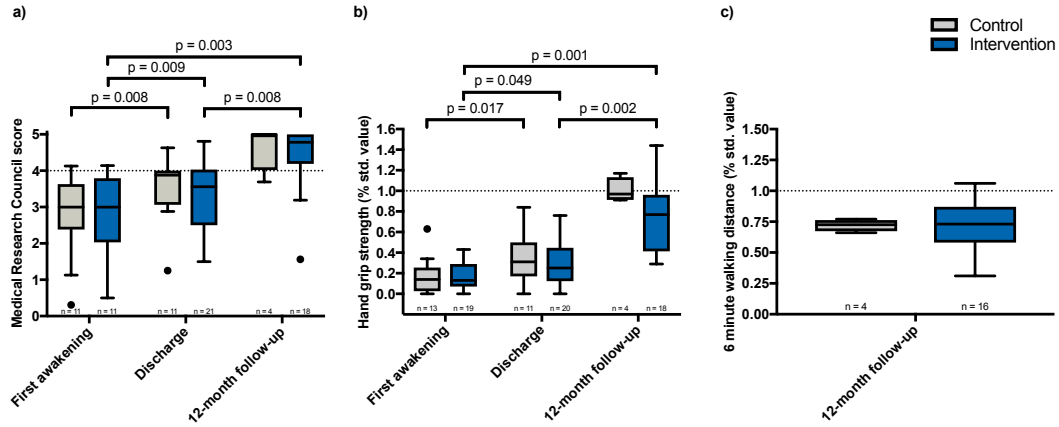

Fig. S1 Muscle strength and functional independence. **a** Medical Research Council score (MRC) increased significantly between first awakening and discharge in both groups. A further increase between discharge and 12-month follo-up could only be observed for the intervention group. The dotted black line indicates an MRC score cut-off value of 4 for ICUAW diagnosis. **b** Relative hand grip strength also increased significantly between first awakening and discharge in both groups while a further increase until 12-month could only be observed for the intervention group. The dotted black line indicates reference values for age and gender matched references. **c** 6 minute walking distance was reduced in both groups at 12-month follow-up. The dotted black line indicates reference values for age and gender matched references. Data are shown as box plots with median and interquartile range. Statistical significance between groups was tested with Mann-Whitney U Test and between timepoints with Wilcoxon-Test.
● represent outliers which are more than 1.5 interquartile ranges above or below the first or third quartile.

Table S4 Fiber type distribution

	Control	Intervention	p-value
Type I	20.5 [16.0/25.3]	23.6 [17.5/26.7]	0.399
Type IIa	46.4 [39.1/63.4]	44.3 [31.7/54.8]	0.140
Type IIb	26.5 [16.7/45.2]	31.7 [19.6/41.6]	0.124

Values represent frequency of fiber types and are presented as median and interquartile range. Statistical significance was tested with Kruskal-Wallis-Test

References

1. Taskforce DAS, Baron R, Binder A, Biniek R, Braune S, Buerkle H, Dall P, Demirakca S, Eckardt R, Eggers V, Eichler I, Fietze I, Freys S, Frund A, Garten L, Gohrbandt B, Harth I, Hartl W, Heppner HJ, Horter J, Huth R, Janssens U, Jungk C, Kaeuper KM, Kessler P, Kleinschmidt S, Kochanek M, Kumpf M, Meiser A, Mueller A, Orth M, Putensen C, Roth B, Schaefer M, Schaefers R, Schellongowski P, Schindler M, Schmitt R, Scholz J, Schroeder S, Schwarzmann G, Spies C, Stingele R, Tonner P, Trieschmann U, Tryba M, Wappler F, Waydhas C, Weiss B, Weisshaar G, (2015) Evidence and consensus based guideline for the management of delirium, analgesia, and sedation in intensive care medicine. Revision 2015 (DAS-Guideline 2015) - short version. Ger Med Sci 13: Doc19

2. Spies C, Kastrup M, Thoralf K, Melzer-Gratzke C, Zielke H, Wolfgang K (2014) SOPs in Intensivmedizin Und Notfallmedizin: Alle Relevanten Standards Und Techniken Für Die Klinik. Thieme,

3. Schonhofer B, Geiseler J, Dellweg D, Moerer O, Barchfeld T, Fuchs H, Karg O, Rosseau S, Sitter H, Weber-Carstens S, Westhoff M, Windisch W, (2014) [Prolonged weaning: S2k-guideline published by the German Respiratory Society]. Pneumologie 68: 19-75

4. De Jonghe B, Sharshar T, Lefaucheur JP, Authier FJ, Durand-Zaleski I, Boussarsar M, Cerf C, Renaud E, Mesrati F, Carlet J, Raphael JC, Outin H, Bastuji-Garin S, Groupe de Reflexion et d'Etude des Neuromyopathies en R, (2002) Paresis acquired in the intensive care unit: a prospective multicenter study. JAMA 288: 2859-2867

5. Wollersheim T, Woehlecke J, Krebs M, Hamati J, Lodka D, Luther-Schroeder A, Langhans C, Haas K, Radtke T, Kleber C, Spies C, Labeit S, Schuelke M, Spuler S, Spranger J, Weber-Carstens S, Fielitz J, (2014) Dynamics of myosin degradation in intensive care unit-acquired weakness during severe critical illness. Intensive Care Med 40: 528-538

6. Bierbrauer J, Koch S, Olbricht C, Hamati J, Lodka D, Schneider J, Luther-Schroder A, Kleber C, Faust K, Wiesener S, Spies CD, Spranger J, Spuler S, Fielitz J, Weber-Carstens S,

(2012) Early type II fiber atrophy in intensive care unit patients with nonexcitable muscle membrane. Crit Care Med 40: 647-650

7. Langhans C, Weber-Carstens S, Schmidt F, Hamati J, Kny M, Zhu X, Wollersheim T, Koch S, Krebs M, Schulz H, Lodka D, Saar K, Labeit S, Spies C, Hubner N, Spranger J, Spuler S, Boschmann M, Dittmar G, Butler-Browne G, Mouly V, Fielitz J, (2014) Inflammation-induced acute phase response in skeletal muscle and critical illness myopathy. PLoS One 9: e92048

8. Huang N, Kny M, Riediger F, Busch K, Schmidt S, Luft FC, Slevogt H, Fielitz J, (2017) Deletion of Nlrp3 protects from inflammation-induced skeletal muscle atrophy. Intensive Care Med Exp 5: 3

9. Zhu X, Kny M, Schmidt F, Hahn A, Wollersheim T, Kleber C, Weber-Carstens S, Fielitz J, (2017) Secreted Frizzled-Related Protein 2 and Inflammation-Induced Skeletal Muscle Atrophy. Crit Care Med 45: e169-e183

10. Weber-Carstens S, Schneider J, Wollersheim T, Assmann A, Bierbrauer J, Marg A, Al Hasani H, Chadt A, Wenzel K, Koch S, Fielitz J, Kleber C, Faust K, Mai K, Spies CD, Luft FC, Boschmann M, Spranger J, Spuler S, (2013) Critical illness myopathy and GLUT4: significance of insulin and muscle contraction. Am J Respir Crit Care Med 187: 387-396