ROLE OF NEUROMUSCULAR ELECTRICAL STIMULATION TO PREVENT RESPIRATORY MUSCLE WEAKNESS IN CRITICALLY ILL PATIENTS AND ITS ASSOCIATION TO CHANGES IN MYOKINES PROFILE. A RANDOMIZED CLINICAL TRIAL.

BY

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What a journey we lived! An entire life, full of colors, including gray.

In just a blink of an eye...

This is just starting…
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ABBREVIATIONS

AHT: Arterial hypertension
APACHE: Acute Physiology and Chronic Health disease Classification System
ARDS: acute respiratory distress syndrome
BDNF: Brain Derived Neurotrophic Factor
BMI: Body mass index
CG: Control Group
cmH2O: centimeters of water
COPD: Chronic obstructive pulmonary disease
C-RP: C-Reactive Protein
DM: Diabetes Mellitus
D1: Day 1
D3: Day 3
D4: Day 4
DTdi: Diaphragmatic thickening fraction
EET: End Expiratory thickness.
EIT: End Inspiratory thickness.
F: Female
FGF21: fibroblast growth factor 21
FiO2: Fraction Inspired of Oxygen
GDF-15: Growth Differentiation Factor 15
Hz: Hertz
ICU: Intensive Care Unit.
ICU-AW: Intensive Care Unit Acquired Weakness
ID NCT: Clinical Trial Identification Number
IL-1: Interleukin 1.
IL-6: Interleukin 6.
IL-10: Interleukin 10.
IL-15: Interleukin 15
IQR: interquartile range
Kg: kilograms
m: Metres
M: Males
mA: Micro amperes
mEq/L: milliequivalents per litre.
MLT: Muscle Layer Thickness
M-mode: Motion Mode
MRC: Medical Research Council
mRNA: micro–Ribonucleic Acid
MSTN: Myostatin
MV: Mechanical Ventilation
mm: millimeters
mmHg: millimeters of mercury
NED: neuromuscular electrophysiological disorders
NMB: Neuromuscular blockade
NMES: Neuromuscular Electrical Stimulation
PC: Pressure Control
pg ml⁻¹: Picograms per milliliter
Ptr, tw: Tracheal Pressure during twitch maneuver
Pts: Points
kg/f: Kilograms per force unit
kg/m²: Kilograms per square meter
RF: Rectus femoris
SAS: Sedation Agitation Scale
SD: Standard Deviation
T₀: baseline (before intervention)
T1: Time 1 (at the end of the intervention)
T2: Time 2 (after two hours from the intervention)
T6: Time 6 (after six hours from the intervention)
Tdi,pi: Diaphragmatic thickness at the peak of twitch inspiration
Tdi,ee: Diaphragmatic thickness at the end of expiration
DTdi: Diaphragmatic thickness at the end of inspiration
TFdi: Diaphragmatic thickening fraction
VI: Vastus Intermedius
VC: Volume control
TNF-alpha: Tumor Necrosis Factor alpha
μsec: Microseconds
ABSTRACT

**Introduction:** Critically ill patients hospitalized at Intensive Care Units (ICU) are characterized by an accelerated muscle wasting, particularly of respiratory muscles, occurring early due to mechanical ventilation (MV). Although active muscle activation may prevent these alterations, it is usually not available at early stages of care because of sedation, favoring a vicious circle. Neuromuscular electrical stimulation (NMES) represents an alternative to achieve muscle contraction in this setting, being able to prevent local muscle wasting, and according to some reports, has the potential to shorten MV time. It has been suggested that this potential benefit might be explained by systemic effects of NMES on distant muscles due to the release of myokines, a diverse range of chemokines secreted by myocytes during contraction. However, no studies have evaluated whether NMES applied to peripheral muscles (quadriceps) in critically ill patients can exert distant muscle effects over the diaphragm, and if such effects are associated to changes in myokine concentrations.

**Objective:** To determine the effects of NMES applied to both quadriceps on myokine plasmatic concentrations, and on peripheral and respiratory muscle function and structure, in mechanical ventilated ICU patients when initiated at an early phase of their critical illness.

**Methods:** Exploratory randomized controlled trial of NMES applied to both quadriceps, twice a day, for 3 days, in comparison to standard care (control group, CG). For myokine characterization (IL-6, BDNF, Myostatin and Decorin), blood samples were obtained at baseline (T0), at the end of the NMES session (T1), and 2 and 6 hours later (T2 and T6). This sampling was repeated on days 1 and 3. For the control group (CG) blood samples were obtained only at T0 and T6. An additional blood sample was also taken on Day 4 (T0) for both groups. Muscle characterization was performed at days 1 and 3 (T0 and T6 respectively). This consisted in ultrasonography of quadriceps muscle layer thickness (MLT), and diaphragmatic thickening fraction (TFdi), along with tracheal tube pressure derived from phrenic nerve magnetic stimulation (Ptr,tw), for diaphragmatic function.
**Results:** 11 patients were randomized: 6 to CG and 5 to NMES. No differences were observed between groups at baseline. No significant interaction was detected between time (across the 4-day protocol) and intervention (NMES or not) for quadriceps MLT change (p-value of 0.12). However, time as factor had a significant impact on MLT explained by a decrease from 1.92 ± 0.81 cm on day 1 to 1.63 ± 0.85 cm on day 3 in the CG, with a p-value of 0.003, while no change along time was observed in the NMES group (Change from 1.76 ± 0.62 cm on day 1 to 1.66 ± 0.61 cm on day 3, with a p-value of 0.51). Concerning diaphragmatic thickening fraction (TFdi), a significant interaction was detected between time (across the 4-day protocol) and intervention (NMES or not) (p-value of 0.006). While in the CG there was an absolute TFdi decrease of 8.93% ± 6.4 (-32.6 ± 25.3 % of relative change) along time, in the NMES group TFdi increased 5.14± 6.55 % (+38.15 ± 58.6 % of relative change). Considering Twitch tracheal pressure (Ptr,tw), a significant interaction was detected between time (across the 4-day protocol) and intervention (NMES or not) (p-value of 0.04). In the control group, Ptr,tw exhibited an absolute change of -1.43 ± 0.68 cmH2O, corresponding to a relative decrease of 19.49% ± 16.98 from baseline values to day 3, while the NMES group experienced an absolute change of +2.5 ± 3.8 cmH2O, equivalent to a relative increase of 46.4 ± 45.6 %. Analyzing the raw plasmatic concentrations of myokines, no significant interaction was detected between time (across the 4-day protocol) and intervention (NMES or not) for any of the myokine concentrations (Decorin, Myostatin, IL-6 and BDNF). Moreover, there were no significant changes observed either within or between groups at any time point.

**Conclusion:** The preliminary data analysed supports the notion that peripheral NMES can preserve respiratory muscle function. It appears that this effect is not mediated by changes in any of the myokines included in the present study. Therefore, alternative mechanisms should be considered to explain how NMES may favour respiratory muscle preservation. The results observed on peripheral muscle layer thickness are yet unconclusive with the limited sample size analysed. Data from a larger number of patients is required to confirm these preliminary conclusions.
INTRODUCTION

Millions of individuals are admitted to intensive care units (ICU) each year. The demand for critical beds at ICUs has been rising for the last few years because of longer life expectancy, chronic diseases, demographic changes and novel disease attacks such as the recent worldwide Covid-19 pandemics (1). Advances in the treatment of critically ill patients has led to an increased survival rate. However, ICU survivors will experience significant consequences, such as an accelerated skeletal muscle wasting derived from the usual context of ICU care, where patients suffer from sepsis and multiple organ failure as well as inactivation and bed-ridden for a prolonged period, particularly during treatment with mechanical ventilation (MV) and sedation (2).

Muscle weakness affecting both the respiratory muscles and peripheral muscles of the axial skeleton is thought to be the key mediator for disability after critical illness (1). The development of ICU-Acquired weakness (ICU-AW), a neuromuscular disorder characterized by profound peripheral muscle weakness and loss of physical functions even after discharge (2–5), is associated with delayed weaning from MV, prolonged ICU and hospital stay, and increased mortality (6–9). Particularly, respiratory muscle weakness (including whole set of inspiratory muscles) will occur in up to 60% of mechanically ventilated patients, being an independent risk factor for prolonged weaning from MV and increased mortality (2,3,10,11).

Diaphragmatic injury and atrophy occur rapidly and frequently in critically ill patients during MV (up to 80%)(12,13). After 18 to 69 hours of diaphragmatic inactivity due to MV, marked atrophy of diaphragm myofibers has been demonstrated (14), along with diaphragmatic muscle weakness, characterized trough the inability to generate normal levels of strength (low inspiratory negative airway pressure deflections) (15–17) or limited diaphragmatic thickness changes during inspiration (low diaphragmatic thickening fraction measured with ultrasound) (16,18–20). All mechanically ventilated patients will experience a significant reduction on their diaphragmatic strength after 3-4
days of MV (12), being associated with prolonged ICU length of stay, longer use of MV (12,13) and a higher risk of complications (10,21).

Risk factors for muscle wasting are believed to be multifactorial, including sepsis, hyperglycemia, multiorgan system failure, use of corticosteroids, neuromuscular blockers, persistent systemic sepsis and muscle inactivity, being the latter one of the most reported one (2,14,17,22–25). In this context, early mobilization of patients in the ICU has become established as an evidence-based strategy to reduce muscle dysfunction (2). But for this strategy to be successful, special attention should be addressed to minimize the use of sedation since failure to awake is the most frequent barrier to achieve a proper muscle activation (1,26). However, physical exercise has limitations in early stages of care where sedation and MV are needed to control respiratory effort and avoid adverse effects secondary to increased oxygen consumption. These considerations determine that muscle activation is usually delayed to later stages of disease, favoring a vicious circle that determines prolonged use of respiratory support and ICU stay (1,16,27,28).

In this scenario, neuromuscular electrical stimulation (NMES), used to induce active muscle contraction in non-cooperative patients, may counteract the early and accelerated muscle wasting, preventing its atrophy (26,29–32). A study in 2019 systematically reviewed the evidence for interventions aimed to prevent sarcopenia in critically ill patients, reporting that NMES was frequently indicated as an effective intervention to preserve muscle mass (33). Gerovasili et al, using ultrasonography, showed in twenty-six critically ill patients with MV, that NMES is well tolerated and seems to preserve the cross-sectional muscle diameter of the vastus intermedius and rectus femoris of quadriceps muscle, being safe and potentially useful for preventing muscle wasting (34). Rodriguez et al applied NMES at one body side of septic patients with MV, finding a significantly higher strength measured trough Medical Research Counsil (MRC) scale on the stimulated side, showing additional benefits over muscle function (35).

Similarly, Routsi et al also showed that NMES could prevent ICU AW in critically ill patients, determining a significantly higher MRC score in the NMES group compared to control group [58 (33 to 60) vs. 52 (2 to 60) respectively, median (range), P =
Interestingly, the weaning period was statistically shorter for the NMES group versus the control group [1 (0 to 10) days vs. 3 (0 to 44) days, respectively, median (range), P = 0.003], suggesting a relation between peripheral NMES and respiratory muscle performance (27). A relation also proposed by Abu-Khaber and cols., who suggested that NMES could prevent the occurrence of ICU AW in all critically ill mechanically ventilated patients, minimizing the degree of muscular weakness, and being able to facilitate weaning from MV (36).

According to some authors, one explanation to this phenomenon is related to the secretion of several systemic factors derived from myocyte activation, the so called myokines. These represent a diverse range of cytokines and chemokines which participate actively in cell-to-cell and organ-to-organ cross-talk, being able to modulate the function and metabolism of distant organs, such as adipose tissue, liver, and brain, both in health and disease (37,38). Muscle contraction, even initiated by NMES, has the potential to counteract muscle degradation not only locally (of peripheral activated muscles), but also systemically through myokines, some of them able to promote an anti-inflammatory and muscle regeneration profile (38,39). The muscle activated by NMES may act as an endocrine organ promoting myokine’s secretion to the circulation, possibly targeting distant muscles (38,40,41). Secretome-based analysis of human myocyte culture medium has revealed over 600 myokines (42,43). However, there is lack of evidence on their biological activity and function for most of them, being only few of them properly characterized and implicated in muscle cell proliferation, differentiation, and growth, especially in pathophysiological conditions such as atrophy or weakness (43). Current knowledge is focused on myokines released directly by muscle contraction such as Myostatin, IL-6, brain derived neurotrophic factor (BDNF), IL-15, Decorin, fibroblast growth factor 21 (FGF21), Myonectin and Irisin (38,40–43).

As mentioned above, some authors have linked NMES application over quadriceps with less weaning time, suggesting a systemic effect of NMES over distant muscles through myokines (27). However as far as we know there isn’t any study focused on this relation, existing scattered evidence for the NMES’s role over myokine secretion in
different settings. For example, it has been reported that NMES in comparison to voluntary exercise, induced a larger increase in serum BDNF (related to muscle regeneration) in healthy males (44). Studying a group of 24 patients with severe and moderate cardiac heart failure, Karavidas et al showed the impact of NMES on peripheral blood markers of immune activation and inflammation, in which patients randomly assigned to a 6-week training program of NMES, reported a significant reduction (–11.5±8.9%) of TNF-alpha and an increase in the IL-10/ TNF-a ratio (37.1 ±29.4%), suggesting the promotion of an anti-inflammatory profile (45). Similarly, Truong et al showed in nine healthy subjects, that 30 min of NMES induced a significant increase in IL-6 from the mean pre-NMES value [0.65 (0.89) to 1.04 (0.89) pg ml\(^{-1}\), \(P = 0.001\)], and a significant decrease in IL-1 [0.08 (0.07) to 0.02 (0.02) pg ml\(^{-1}\), \(P = 0.041\)] and TNF-a [2.42 (0.54) to 2.16 (0.59) pg ml\(^{-1}\), \(P = 0.021\)] (46).

As we established previously, critical illness (sedation, immobilization, inflammatory insults and MV) accelerates muscle wasting, leading to peripheral and respiratory muscle weakness. The latter being faster and determining several negative clinical implications such as delayed weaning and prolonged MV. Early muscle contraction may contribute to prevent these negative consequences. However, exercise is limited at early stages of disease as sedation is frequently needed, and few strategies have been proved to counteract critical factors related to an accelerated muscle wasting such as unloading and inflammation. In this scenario, there is evidence which suggests a potential role of NMES in preventing muscle loss and disfunction, favoring weaning from MV. In the present thesis we propose that this effect may be mediated by secretion of myokines from peripheral muscles, in response to NMES application, which may exert effects on distant muscles.
HYPOTHESIS

In mechanical ventilated ICU patients, quadriceps NMES contributes to prevent respiratory muscle weakness when initiated at an early phase of critical illness, and this effect is associated to acute changes in myokine profile.

GOALS

General goal
To determine the effects of quadriceps NMES in mechanical ventilated ICU patients on myokine plasmatic concentrations, peripheral and respiratory muscle function, and structure, when initiated at an early phase of their critical illness.

Specific goals

1) To determine the effects of a 3-day quadriceps NMES protocol on muscle layer thickness of vastus intermedius and rectus femoris of the quadriceps (measured with ultrasonography)

2) To determine the effects of a 3-day quadriceps NMES protocol on diaphragmatic function (diaphragmatic thickening fraction measured with ultrasonography and transdiaphragmatic twitch pressure)

3) To determine the acute effect of a quadriceps NMES session on myokine plasmatic concentrations (IL-1, IL-6, IL-15, BDNF, Myostatin and Decorin)
METHODOLOGY

Study design.

This report comprises a preliminary analysis from a randomized controlled study started in June 2022 in the ICU of the Hospital clínico UC-CHRISTUS, Santiago, Chile (recruitment currently active). The research protocol was approved by the central ethics committee of the Metropolitan region in February 2022 (Figure S4), and by the regional secretary of health (SEREMI) in May 2022 (recruitment started). Written informed consent was obtained from the patients’ relatives, which was confirmed afterwards by those patients able to consent after extubation (re-consent). The study protocol was registered into a clinical trials platform (ClinicalTrials.gov) with the ID NCT05536531.

Eligibility criteria

Inclusion criteria: Adult patients ≥ 18 years old admitted to the ICU, in a Sedation-non cooperative state (SAS 1 or 2), connected to invasive MV within the first 24-72 hours, with risk factors for ICU-AW (diagnosis of sepsis, hyperglycemia, APACHE II admission score >13 pts, use of corticosteroids, and/or muscle inactivity due to deep sedation), and with informed consent provided by surrogate (legal tutor).

Exclusion criteria: Pregnancy, obesity (Body Mass Index >35 kg/m2), pre-existing neuromuscular diseases (e.g. myasthenia Gravis, Guillain-Barré disease), diagnosis of end-stage malignancy, diseases with systemic vascular involvement such as systemic lupus erythematosus, use of neuromuscular blockers, cardiac pacemakers, or presence of technical obstacles for NMES implementation such as bone fractures, skin lesions, or severe local edema (3 to 4 fovea sign) in lower extremities.
Protocol overview.

The intervention group received during three days, a twice a day NMES session plus standard care, in comparison to control group (CG), which received only standard care (no NMES). Muscle characterization of quadriceps and diaphragm (ultrasonography evaluation of muscle layer thickness for quadriceps and diaphragmatic thickening fraction and tracheal tube pressure assessment, derived from magnetic stimulation of phrenic nerve, for diaphragmatic function) were performed at baseline (Day 1, prior to the first NMES session) and at day 3 of NMES (after the first NMES session of day 3). Myokine measurements (IL-6, BDNF, Myostatin and Decorin), through blood serum obtained from peripheric blood samples, were performed at baseline, just before starting NMES (T₀), at the end of NMES session (T₁), and 2 and 6 hours later (T₂ and T₆). This myokine curves were repeated on days 1 and 3 at the first NMES session of the day, while for the control group blood samples for myokine analysis were collected on days 1 and 3 but limited to 2 samples separated by 6 hours (as a parallel to T₀ and T₆ from the NMES group). For both groups, an additional single blood sample for Myokine measurement was performed on day 4, in the early morning (T₀).

Methodology according to each specific goal

1. To determine the effects of a 3-day NMES protocol on muscle layer thickness of vastus intermedius and rectus femoris of the quadriceps (measured with ultrasonography): Muscle layer thickness (MLT) of the quadriceps muscle [vastus intermedius (VI) and rectus femoris (RF)] were bilaterally measured by high-resolution real-time ultrasonography at the midpoint between the anterior superior iliac spine and the upper pole of the patella, with the patient in a supine position and the legs relaxed lying flat in extension. MLT is calculated as the mean of 2 measurements on each leg. Additionally, VI and RF thickness are measured independently. All examinations were
performed by the same operator, using a 10 MHz linear transducer connected to a portable ultrasound system (Mindray HDI-1000, ATL©, Bothell, USA).

2. To determine the effects of a 3-day NMES protocol on diaphragmatic function (diaphragm thickening fraction measured with ultrasonography and twitch tracheal tube pressure)

2.1. NMES session protocol: NMES was applied twice a day simultaneously on quadriceps muscles of both lower limbs using a commercial electrical stimulator (TRAINFES® 6 ADVANCED, Biomedical devices Spa, Santiago, Chile). Four rubber surface electrodes were placed over motor points of each quadriceps. However, since the surface of electrodes covered big proportion of muscle surface, anatomical distribution of the belly muscle plus visible contraction of it was considered for correct setting. The stimulation was delivered by biphasic current, symmetric (compensated) impulses of 45-50 Hz frequency, and 400 μsec of pulse duration. The stimulus lasted 25 minutes, with an on/off programming of 5 seconds on/5 seconds off, at current intensities able to cause maximal visible contractions. If necessary, intensity (milliamperes) was adjusted every 3 minutes to maintain visible contractions.

2.2. Twitch manoeuvre was performed using a MAGSTIM 200 device, which provided magnetic stimulus over the cervical emergence of phrenic nerves (C6-C7) through a single coil approach. At least 3 twitch stimuli were provided at 100% of intensity (maximum of 2 Tesla). One operator held the coil, and the activation safety buttons on it, and discharged the magnetic stimuli by a foot pedal. A second operator performed simultaneously the ultrasonography of the diaphragm.

2.3. Diaphragm thickening fraction (TFdi) was defined as the percentage change in diaphragm thickness during peak twitch inspiration [quotient of Diaphragmatic thickness during inspiration (DTdi) and thickness at end expiration (Tdi,ee)]. DTdi was calculated from the difference between Tdi,ee and thickness at peak twitch inspiration (Tdi,pi). To obtain these values during twitch manoeuvre, real-time diaphragmatic thickness change was recorded prospectively during M-mode ultrasonography (with a 10
MHz linear transducer) during at least three twitch manoeuvres (inspiratory effort induced by magnetic stimulation). The transducer was set longitudinally between 8th-10th intercostal space on the antero-lateral axillar line (17,47,48)

2.4. **Tracheal tube pressure** was simultaneously measured during twitch manoeuvre (Ptr,tw). For this purpose, a three-way airway valve (closed system) was connected inline into patient’s mechanical ventilation circuit, next to the end of the endotracheal tube. This valve had a pressure port which allowed a connection from a pneumotachograph to register pressure curves over time, being able to identify pressure deflection in response to the twitch manoeuvre during a transient occlusion of the circuit with the valve. This valve is not the standard procedure but was added in the present study as an innovation to avoid mechanical ventilation interaction (including disconnection from it as traditional manoeuvre describes) and the potential underestimation of the pressure change given the rather large volume of the airway circuit and its compliance. Ptr,tw value was considered as the difference between the lower pressure (value of negative spike) during twitch, and the pressure value just before twitch (pause pressure).

3. **To determine the acute effect of a NMES session on myokine plasmatic concentrations (IL-1, IL-6, IL 15, BDNF, Myostatin and Decorin):** All ICU patients had an arterial line for blood samples and arterial pressure monitoring. Peripheral blood samples were obtained from these, drawn into citrated tubes, immediately centrifuged, and frozen (-80º) until analysis. Each sample was coded with a unique identifier to track the participant and timing of blood draws. Technicians responsible for laboratory analysis were blinded to sample’s origin. For measuring BDNF, IL-1, IL-6 and IL-15, a commercially available multiplexed ELISA kit (Human magnetic Luminex screening assay R&D systems from GeneXpress) was used, requiring 25 ul of serum for simultaneous determination of all four markers. Myostatin and serum Decorin concentrations were assessed using a human Decorin and Myostatin ELISA kit. All samples were processed following manufacturer’s protocol.
Statistical analysis.

Sample size calculation and randomization.

There is limited data on strategies preventing diaphragmatic dysfunction in an acute setting. However, based on Jaber et al.’s findings of a significant \( \text{Ptr}_{\text{tw}} \) decrease after 5-6 days of MV, with a mean decrease of 6.3 cmH2O from baseline (12), we anticipated that NMES could partially mitigate this phenomenon by 50%. This would result in an expected mean decrease of 3.15 cmH2O in the NMES group, in contrast to the anticipated 6.3 cmH2O decrease in the control group. Given these assumptions, a standard deviation of 3 cmH2O in both groups, a confidence level of 95%, and a power of 80%, the original calculated sample size needed to detect a significant impact on diaphragm weakness was 32 patients. However, as this original calculation was based on data obtained at a different time frame, and as we had no real data to estimate the potential magnitude of the effect of NMES, we decided to perform an initial analysis after including the first 11 patients.

Statistical analysis (descriptive and inferential)

The data distribution was checked using the Shapiro Wilk test, guiding the appropriate statistical test selection. Continuous variables are presented using either the median and interquartile range (IQR) or the mean and standard deviation (SD), depending on whether the distribution is non-parametric or parametric, respectively. Categorical data is presented as frequencies and their corresponding percentages, its comparisons were made using chi- square test. The statistical analyses were conducted using the GraphPad Software-Prism (version 9.0.1) considering a \( P \)-value <0.05 as the value for statistical significance for all comparisons. Both absolute and relative changes are presented as appropriate. The latter (delta changes) were calculated with respect to baseline values on day one, serving as reference for assessing changes over time.
To compare NMES and CG in all interest outcomes, and considering potential interaction between time and intervention, a two-way ANOVA with a mixed-effects model was applied (due to uneven sample size in each group). Post-hoc analysis was performed as appropriate (Sidak test). Particularly, for the analysis of myokines, common assessment time points were considered for comparison between groups (T₀ and T₆ at day 1 and 3, and T₀ at day 4). For complementary within group analysis, multiple t-tests were performed in case of parametric distribution, while Friedman test was used for non-parametric distribution.
RESULTS

1. Patient characteristics

Eleven patients have been recruited. Six were male (54.5%). The most frequent admission diagnosis was septic shock. The average age of the participants was 53.3 ± 14.7 years, with an average BMI of 24.8 ± 3.7 kg/m². At the time of inclusion, the participants had been using mechanical ventilation for an average of 47.6 ± 13.6 hours. Relevant individual characteristics of the patients are presented in Table 1.

Clinical characteristics of the patients randomized to each group are presented in Table 2. Groups appear well balanced for most characteristics except for age where a mean difference of 11.1 years was observed.
### Table 1. Baseline characteristics

<table>
<thead>
<tr>
<th>Number</th>
<th>Group</th>
<th>Sex</th>
<th>Age (years)</th>
<th>BMI (Kg/m²)</th>
<th>Hours on MV</th>
<th>Main diagnostic</th>
<th>APACHE II</th>
<th>Charlson index</th>
<th>Main Comorbidities</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>M</td>
<td>35</td>
<td>30</td>
<td>36</td>
<td>Traumatic Brain injury</td>
<td>13</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>M</td>
<td>54</td>
<td>19.6</td>
<td>48</td>
<td>Pneumocystis Jiroveci Pneumonia</td>
<td>32</td>
<td>3</td>
<td>Lymphoproliferative Syndrome, No Hodgkin Lymphoma and Acute Kidney Injury</td>
</tr>
<tr>
<td>6</td>
<td>Control</td>
<td>F</td>
<td>62</td>
<td>26.6</td>
<td>48</td>
<td>Septic Shock</td>
<td>28</td>
<td>4</td>
<td>Hepatic disease and peripheral vascular disease</td>
</tr>
<tr>
<td>8</td>
<td>Control</td>
<td>F</td>
<td>42</td>
<td>22.8</td>
<td>24</td>
<td>ARDS</td>
<td>27</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>9</td>
<td>Control</td>
<td>M</td>
<td>66</td>
<td>26.7</td>
<td>48</td>
<td>Septic Shock</td>
<td>29</td>
<td>3</td>
<td>Lymphoma, AHT, DM and Chronic Kidney Injury</td>
</tr>
<tr>
<td>11</td>
<td>Control</td>
<td>F</td>
<td>61</td>
<td>26.3</td>
<td>48</td>
<td>Drug intoxication</td>
<td>18</td>
<td>0</td>
<td>Psychiatric disorder</td>
</tr>
<tr>
<td>3</td>
<td>NMES</td>
<td>F</td>
<td>23</td>
<td>24.8</td>
<td>48</td>
<td>Hepatic failure due to drug intoxication</td>
<td>27</td>
<td>0</td>
<td>Psychiatric disorder, AHT</td>
</tr>
<tr>
<td>4</td>
<td>NMES</td>
<td>M</td>
<td>67</td>
<td>22.5</td>
<td>68</td>
<td>Acute Renal failure</td>
<td>24</td>
<td>2</td>
<td>AHT, DM, Myeloma suspect</td>
</tr>
<tr>
<td>5</td>
<td>NMES</td>
<td>F</td>
<td>49</td>
<td>17.8</td>
<td>36</td>
<td>Hepatic failure</td>
<td>29</td>
<td>5</td>
<td>No Hodgkin Lymphoma and Acute Kidney Injury, DM</td>
</tr>
<tr>
<td>7</td>
<td>NMES</td>
<td>M</td>
<td>66</td>
<td>27.8</td>
<td>48</td>
<td>Septic Shock</td>
<td>30</td>
<td>1</td>
<td>Neoplasm</td>
</tr>
<tr>
<td>10</td>
<td>NMES</td>
<td>M</td>
<td>64</td>
<td>27.7</td>
<td>72</td>
<td>COVID-19 ARDS</td>
<td>23</td>
<td>2</td>
<td>Mantle Cell Lymphoma, AHT</td>
</tr>
</tbody>
</table>

**Total** | 6 M (54.5%) | 53.6 ±14.7 | 24.8 ± 3.7 | 47.6 ± 13.6 | - | 27 (23-29) | 2 (0-3) | - |

DM: Diabetes Mellitus; AHT: Arterial hypertension; Kg: kilograms; m: Metres; NMES: Neuromuscular electrical stimulation; M: Male; F: Female; ARDS: acute respiratory distress syndrome. Categorical data is presents as frequency (percentage) while numeric variables are summarized in total as either mean (SD) or Median (IQR) as appropriate according to distribution.
Table 2. Clinical characteristics by intervention group

<table>
<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>NMES group (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline characteristics</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>53.3 ± 21.15</td>
<td>64.4 ± 23.66</td>
</tr>
<tr>
<td>Gender (male/total)</td>
<td>3/6 (50%)</td>
<td>3/5 (54.5%)</td>
</tr>
<tr>
<td>BMI (Kg/m2)</td>
<td>25.3 ± 3.62</td>
<td>24.1 ± 4.17</td>
</tr>
<tr>
<td><strong>Severity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>APACHE II</td>
<td>24.5 ± 7.34</td>
<td>26.6 ± 3.05</td>
</tr>
<tr>
<td>Charlson index</td>
<td>1.67 ± 1.86</td>
<td>2 ± 1.87</td>
</tr>
<tr>
<td>Vasoactive drugs (users/total)</td>
<td>4/6 (67%)</td>
<td>3/5 (60%)</td>
</tr>
<tr>
<td>Prior NMB use (users/total)</td>
<td>1/6 (17%)</td>
<td>2/5 (40%)</td>
</tr>
<tr>
<td>Prone position (users/total)</td>
<td>0/6 (0%)</td>
<td>1/5 (20%)</td>
</tr>
<tr>
<td><strong>Diagnosis at admission</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory (%)</td>
<td>2 (33)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Sepsis (%)</td>
<td>2 (33)</td>
<td>1 (20)</td>
</tr>
<tr>
<td>Trauma (%)</td>
<td>1 (17)</td>
<td>-</td>
</tr>
<tr>
<td>Metabolic (%)</td>
<td>1 (17)</td>
<td>3 (60)</td>
</tr>
<tr>
<td><strong>MV use at inclusion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Time on MV (hours)</td>
<td>42 ± 10.04</td>
<td>54 ± 15.13</td>
</tr>
<tr>
<td>Mode: PC/total (%)</td>
<td>4/6 (67%)</td>
<td>4/5 (83%)</td>
</tr>
<tr>
<td>VC/total (%)</td>
<td>2/6 (33%)</td>
<td>1/5 (17%)</td>
</tr>
<tr>
<td>PEEP level (cmH₂O)</td>
<td>6 ± 2</td>
<td>8 ± 3</td>
</tr>
<tr>
<td>FiO2 (%)</td>
<td>27 ± 3</td>
<td>34 ± 6</td>
</tr>
<tr>
<td>Oxygenation (PaO₂/FiO₂ ratio)</td>
<td>397.7 ± 93.9</td>
<td>341.6 ± 112.7</td>
</tr>
<tr>
<td><strong>Metabolic variables</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium (mEq/L)</td>
<td>4.7 ± 0.8</td>
<td>3.8 ± 0.3</td>
</tr>
<tr>
<td>Sodium (mEq/L)</td>
<td>138 ± 3</td>
<td>143 ± 9</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.75 ± 0.98</td>
<td>1.7 ± 1</td>
</tr>
<tr>
<td>Blood Urea Nitrogen (mg/dL)</td>
<td>38 ± 31</td>
<td>41 ± 17</td>
</tr>
<tr>
<td><strong>Muscle response factors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluid balance (ml)</td>
<td>1703 ± 819</td>
<td>1012 ± 1121</td>
</tr>
<tr>
<td>Capillary response time (sec)</td>
<td>1 (1-2)</td>
<td>1 (1-2)</td>
</tr>
<tr>
<td>Edema (Fovea sign level)</td>
<td>1 (1-2)</td>
<td>1</td>
</tr>
<tr>
<td>Inflammation, C-RP (mg/dL)</td>
<td>9.6 (7.8-17.6)</td>
<td>6.1 (3.5 – 23.4)</td>
</tr>
<tr>
<td>Infection, Leukocytes (10³/μL)</td>
<td>6100 ± 1931</td>
<td>8520 ± 7682</td>
</tr>
<tr>
<td><strong>Clinical outcomes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total time on MV [days; Median (IQR)]</td>
<td>5 (3-9.5)</td>
<td>6 (3-30)</td>
</tr>
<tr>
<td>ICU length of stay (days; Mean ± SD)</td>
<td>14.5 ± 4.7</td>
<td>20.8 ± 13.5</td>
</tr>
<tr>
<td>Hospital length of stay (days; Mean ± SD)</td>
<td>52.5 ± 40.7</td>
<td>20.8 ± 13.7</td>
</tr>
<tr>
<td>Vital status at ICU discharge (Alive, total)</td>
<td>5/6 (83.3%)</td>
<td>5/5 (100%)</td>
</tr>
<tr>
<td>Vital status at hospital discharge (Alive/total)</td>
<td>5/6 (83.3%)</td>
<td>5/5 (100%)</td>
</tr>
</tbody>
</table>

Values are given as n, %, Median and IQR, Mean and standard deviation as appropriate. NMES: Neuromuscular electrical stimulation; BMI: Body Mass Index; MV: Mechanical ventilation; APACHE II: Acute Physiology and Chronic Health disease Classification System II; C-RP: C-Reactive Protein; FiO2: Fraction Inspired of Oxygen; PC: Pressure Control; VC: Volume control; NMB: Neuromuscular blockade; ICU: Intensive Care Unit.
2- Effect of a 3-day NMES protocol on muscle layer thickness of the quadriceps (measured with ultrasonography)

Concerning peripheral muscle wasting (specifically the quadriceps muscle), no significant interaction was detected between time (across the 4-day protocol) and intervention (NMES or not) for quadriceps MLT (p-value of 0.12) (Figure 1). Nor was any difference observed for NMES and CG when analyzing the rectus femoris (p-value of 0.16 and 0.19 respectively) and the vastus intermedius (p-value of 0.47 and 0.56 respectively) in a separate manner. Individual data by side and muscle group are provided on table S1.

Time as factor had a significant impact on MLT explained by a decrease from 1.92 ± 0.81 cm on day 1 to 1.63 ± 0.85 cm on day 3 in the CG, with a p-value of 0.003, while no change along time was observed in the NMES (Change from 1.76 ± 0.62 cm on day 1 to 1.66 ± 0.61 cm on day 3, with a p-value of 0.51). Factors potentially related to muscle response to NMES are provided in table S2.

Figure 1. Quadriceps muscle layer thickness. In red lines, change in muscle layer thickness from day 1 to 3 (both legs combined), for the neuromuscular electrical stimulation group (NMES). In black lines, mean values for quadriceps muscle layer thickness in control group, also from day 1 to 3, both legs combined. Values are expressed as mean and standard deviation. P-value corresponds to the interaction between intervention and time from a Two-way ANOVA mixed model analysis.

* Corresponds to a p-value of 0.003 on MLT decrease for CG, calculated with a Sidak’s post hoc analysis.
3- Effect of a 3-day NMES protocol on diaphragmatic function (diaphragm thickening fraction and tracheal twitch pressure)

Concerning diaphragmatic thickening fraction (TFdi), a significant interaction was detected between time (across the 4-day protocol) and intervention (NMES or not) (p-value of 0.006). While in the CG there was an absolute TFdi decrease of 8.93 ± 6.4 (-32.6 ± 25.3 % of relative change) along time, in the NMES group TFdi increased 5.14 ± 6.55 (+38.15 ± 58.6 % of relative change) (Table 3, Figure 2).

<table>
<thead>
<tr>
<th>Table 3. Diaphragmatic thickening fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td>9</td>
</tr>
<tr>
<td>11</td>
</tr>
<tr>
<td>Mean ± SD</td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>Mean ± SD</td>
</tr>
</tbody>
</table>

EIT: End Inspiratory Thickness; EET: End Expiratory thickness; TFdi: Diaphragmatic Thickening fraction; C: Control; NMES: Neuromuscular Electrical Stimulation. Negative values of change imply a decrease from baseline values. EIT and EET values corresponded to the mean value calculated from at least 2 attempts of measurement.
Considering Twitch tracheal pressure ($P_{tr, tw}$) as proxy for diaphragmatic function, a significant interaction was detected between time (across the 4-day protocol) and intervention (NMES or not) ($p$-value of 0.04). In the control group, $P_{tr, tw}$ exhibited an absolute change of $-1.43 \pm 0.68 \text{ cmH}_2\text{O}$, corresponding to a relative decrease of $19.49 \pm 16.98\%$ from baseline values to day 3, while the NMES group experienced an absolute change of $+2.5 \pm 3.8 \text{ cmH}_2\text{O}$, equivalent to a relative increase of $46.4 \pm 45.62\%$ (Table 4 and Figures 3 and figure S1).

A representative graphical processing of pressure curves from the pneumotachograph during twitch maneuver is shown in figure S2.
Table 4. Twitch tracheal pressure change

<table>
<thead>
<tr>
<th>Patient</th>
<th>Group</th>
<th>Day 1</th>
<th>Day 3</th>
<th>Intragroup change (From day 1 to 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient</td>
<td>Group</td>
<td>Ptr,tw</td>
<td>Ptr,tw</td>
<td>Absolute change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cmH₂O</td>
<td>cmH₂O</td>
<td>(cmH₂O)</td>
</tr>
<tr>
<td>1</td>
<td>C</td>
<td>11.52</td>
<td>10.53</td>
<td>-0.99</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
<td>9.18</td>
<td>8.15</td>
<td>-1.03</td>
</tr>
<tr>
<td>6</td>
<td>C</td>
<td>4.77</td>
<td>2.31</td>
<td>-2.46</td>
</tr>
<tr>
<td>8</td>
<td>C</td>
<td>7.83</td>
<td>5.87</td>
<td>-1.96</td>
</tr>
<tr>
<td>9</td>
<td>C</td>
<td>9.36</td>
<td>8.73</td>
<td>-0.63</td>
</tr>
<tr>
<td>11</td>
<td>C</td>
<td>10.87</td>
<td>9.37</td>
<td>-1.5</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>8.96 ± 2.43</td>
<td>7.66 ± 3.2</td>
<td>-1.43 ± 0.68</td>
</tr>
<tr>
<td>3</td>
<td>NMES</td>
<td>8.26</td>
<td>16.97</td>
<td>8.71</td>
</tr>
<tr>
<td>4</td>
<td>NMES</td>
<td>2.22</td>
<td>4.02</td>
<td>1.8</td>
</tr>
<tr>
<td>5</td>
<td>NMES</td>
<td>3.25</td>
<td>3.8</td>
<td>0.55</td>
</tr>
<tr>
<td>7</td>
<td>NMES</td>
<td>5.67</td>
<td>7.54</td>
<td>1.87</td>
</tr>
<tr>
<td>10</td>
<td>NMES</td>
<td>11.25</td>
<td>10.75</td>
<td>-0.5</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>6.1 ± 3.7</td>
<td>8.62 ± 5.47</td>
<td>2.5 ± 3.8</td>
</tr>
</tbody>
</table>

Ptr,tw: Twitch Tracheal pressure; C: Control; NMES: Neuromuscular Electrical Stimulation. Negative values of change imply a decrease from baseline values, meanwhile positive values imply an increase from it. Ptr,tw values corresponded to the mean value calculated from at least 2 attempts of measurement.

Figure 3. Twitch tracheal pressure. In red, mean values for twitch tracheal pressure (Ptr,tw) from day 1 to 3, in the neuromuscular electrical stimulation group (NMES). In black lines, median values for Ptr,tw in control group. Values are represented as mean and standard deviation. P-value corresponds to the interaction between intervention and time from a Two-way ANOVA mixed model analysis.
4. Effect of NMES on myokine plasmatic concentrations (IL-6, BDNF, Myostatin and Decorin)

4.1 Effect of NMES on myokine plasmatic concentrations over protocol study period.

Analyzing the raw plasmatic concentrations of myokines, no significant interaction was detected between time (across the 4-day protocol) and intervention (NMES or not) for any of the myokines analyzed; with a p-value of 0.2, 0.43, 0.87 and 0.48 for Decorin, Myostatin, IL-6 and BDNF, respectively (Figures 4, 5, 6, 7). It’s noteworthy that Myostatin plasmatic concentrations for the NMES group illustrate changes only from 3 and 2 patients over day 1 and 3 respectively, meanwhile for the CG only one patient exhibited detectable Myostatin plasmatic concentrations changes (Figure S3).

Analyzing the relative changes (delta) on myokine’s plasmatic concentrations, with respect to baseline values on day one serving as reference, no significant interaction was detected between time (across the 4-day protocol) and intervention (NMES or not) for Decorin (p value 0.64), Myostatin (p value 0.42), BDNF (p value 0.26), nor IL-6 (p value 0.27)

![Figure 4. Decorin plasmatic concentrations over time. Values are shown as median and interquartile range. In red NMES group trends. In black control group trends. D1: Day 1; D3: Day 3; D4: Day 4; T0: Baseline; T1: At the end of NMES session; T2: 2 hours after T1, and T6: 6 hours after T1. P-value corresponds to the interaction between intervention and time from a Two-way ANOVA mixed model analysis.](image1)

![Figure 5. Myostatin plasmatic concentrations over time. Values are shown as median and interquartile range for NMES group in red trends. In black are represented median values for control group. D1: Day 1; D3: Day 3; D4: Day 4; T0: Baseline; T1: At the end of NMES session; T2: 2 hours after T1, and T6: 6 hours after T1. P-value corresponds to the interaction between intervention and time from a Two-way ANOVA mixed model analysis.](image2)
4.2 Acute effect of a single NMES session on myokine plasmatic concentrations

Graphical representation of Decorin, Myostatin, IL-6, and BDNF acute changes in response to NMES are shown in figures 8, 9, 10 and 11, respectively. Concerning myostatin, only 3 patients on day 1, and 2 patients on day 3 exhibited detectable plasmatic concentrations throughout the 6-hour assessment period after NMES session (individual values are shown in figure 9).
Figure 9. **Myostatin acute response to NMES.** Values correspond to Myostatin plasmatic concentrations only for the NMES group. Left graph illustrate changes over day 1, meanwhile right graph shows the changes over day 3. Individual data for each patient with detectable values is presented (thin red lines). No summary trend is shown given the limited number of patients with detectable Myostatin levels. D1: Day 1; D3: Day 3; D4: Day 4; T0: Baseline; T1: At the end of NMES session; T2: 2 hours after T1, and T6: 6 hours after T1. p-value corresponds to the difference calculated from a Friedmann test.

Figure 10. **IL-6 acute response to NMES.** Values correspond to IL-6 plasmatic concentrations only for the NMES group. Left graph illustrate changes over day 1, meanwhile right graph shows the changes over day 3. Individual data for each patient with detectable values is presented (thin red lines), as well as the median (thick red line). D1: Day 1; D3: Day 3; D4: Day 4; T0: Baseline; T1: At the end of NMES session; T2: 2 hours after T1, and T6: 6 hours after T1. p-value corresponds to the difference calculated from a Friedmann test.

Figure 11. **BDNF acute response to NMES.** Values correspond to BDNF plasmatic concentrations only for the NMES group. Left graph illustrate changes over day 1, meanwhile right graph shows the changes over day 3. Individual data for each patient with detectable values is presented (thin red lines), as well as the median (thick red line). D1: Day 1; D3: Day 3; D4: Day 4; T0: Baseline; T1: At the end of NMES session; T2: 2 hours after T1, and T6: 6 hours after T1. p-value corresponds to the difference calculated from a Friedmann test.
DISCUSSION

NMES of the quadriceps appears to have a potential positive impact on diaphragm function, assessed either by the Twitch tracheal pressure or the diaphragmatic thickening fraction obtained during a phrenic twitch stimulation. No clear effect of NMES on quadriceps MLT has been observed. In terms of myokines, we have observed no evidence of an impact of NMES on any of the biomarkers assessed.

Local effects of NMES: Muscle layer thickens changes in response to NMES.

Concerning the local effect of NMES on quadriceps muscle layer thickness, the preliminary analysis presented in this report does not reveal a statistically significant effect of NMES. However, a closer examination of the time dependent changes observed in each group indicates different trends between them. Patients in the control group experienced a significant decrease in quadriceps MLT, as expected according to the anticipated muscle wasting phenomena. In contrast, patients from the NMES group showed no significant change over time.

Several studies in critically ill patients have consistently reported a significant impact of NMES on preventing local muscle atrophy (49–52). However, in those studies NMES was typically applied for several days, whereas we limited the intervention to a duration of 3 days. For instance, Silva and cols. In a study involving patients with consciousness disorders (40 critically ill traumatic brain injury patients), observed similar results at day 3, with no significant interaction between time and intervention (50). However, they detected benefits from the 7th day onward in terms of muscle thickness preservation (specifically, rectus femoris and tibial anterior) and neuromuscular electrophysiological disorder (NED), as measured through evoked peak force. By the 14th day, the control group exhibited a significant reduction in muscle thickness of tibialis anterior and rectus femoris, with a mean reduction of $-0.33$ mm ($-14\%$) and $-0.49$ mm
(− 21%), respectively (p-value < 0.0001), whereas muscle thickness was preserved in the NMES group. Additionally, the control group showed a higher incidence of NED (47% vs. 0% in the NMES group, p-value < 0.0001, risk ratio of 16), while the NMES group demonstrated an increase in the evoked peak force (2.34 kg/f, p-value < 0.0001), contrasting with the decrease observed in the control group (− 1.55 kg/f, p-value < 0.0001). The NMES protocol required at least 7 days to prevent muscle architecture disorders and address weakness, a finding similar to that reported by Viera and cols in the same population, where significant changes in muscle echogenicity and thickness where observed between Day 1 and Day 7 in the control group compared to the NMES group (53).

Additionally, Viera and cols showed significant preservation of muscle quality at day 4 in the NMES group, but no time-dependent changes in muscle thickness, consistent with our own dataset in the post-hoc analysis. Nevertheless, while the later analysis may still be underpowered due to the short intervention duration (3 days protocol) and sample size, thus affecting a definitive conclusion, it’s important to consider some differences regarding the NMES protocol. Firstly, unlike the four small electrodes commonly used and reported by other authors (50,53), we used larger electrodes. The increased surface area of our device secures the stimulation of all nervous roots of the quadriceps muscles, thereby stimulating it in a more comprehensive and synchronous manner. And second, we applied 2 sessions per day, as some authors suggested for better results (52,54).

**Distant effects of NMES: Impact over diaphragmatic function**

Despite the small number of patients analysed, the findings presented in this report support the hypothesis that diaphragmatic function could be better preserved by the early application of NMES over a peripheral muscle such as the quadriceps. Diaphragmatic weakness has been recognized as one of the negative factors contributing to a more difficult weaning of mechanical ventilation, being also an important determinant of poor
outcomes in this population (55–57). Peripheral muscle activation through NMES has been associated with the prevention of ICU AW, recovery of physical function and according to some authors, shortening of weaning time and duration of MV (27,36,49). It has been proposed that the potential impact of NMES on weaning time and duration of MV might be mediated by distant effects of peripheral NMES on respiratory muscles (27). However, up to now no study had directly assessed whether peripheral NMES preserves respiratory muscle function. The present study is the first attempt to test this hypothesis and the results presented are encouraging indicating that peripheral NMES favors diaphragmatic function, measured either by its pressure generating capacity (tracheal pressure during twitch maneuver), or by its contractile response (thickening fraction of the diaphragm during twitch maneuver). However, our original hypothesis was that this effect would be mediated by changes in the concentration of circulating myokines in response to NMES, a mechanism that our current results do not support.

**Systemic changes induced by NMES: does the dose matter?**

In the 11 patients included up to now we observed no differences between the NMES and the control group in any of the 4 myokines analyzed throughout the 4-day study period. Additionally, when analyzing the acute effect of an NMES session within the initial 6 hours, among the five patients allocated to NMES and across the six NMES sessions evaluated (as part of a three-day NMES protocol), no change was observed along time for any of the four myokines measured. While a handful of previous studies in critically ill patients suggested the potential for NMES, either alone or combined with active exercise, to modulate plasmatic concentrations of myokines (53, 54 ), there exists a substantial body of evidence that fails to corroborate this effect (55, 56–60, 61, 62). For instance, Akar et al. demonstrated that NMES could decrease plasmatic levels of IL-6 and IL-8 in mechanically ventilated COPD patients following 20 daily NMES sessions (administrated five times weekly) targeting both upper and lower extremities (specifically,
the deltoid and quadriceps muscle, respectively) (53). However, contradictory outcomes have been reported by other researchers (52)(54) following similar intervention protocols. Notably, significant changes were not observed for any of the muscle growth and inflammatory markers evaluated (Including IGF-1, IL-2, IL-4, IL-6 and TNF-α, among other) following 7 to 14 daily NMES sessions, consistent with the absence of myokine changes found in our study and much of the literature to date.

There has been a growing interest in exploring the potential systemic effects of NMES in the ICU; however, its characterization remains somewhat elusive. Over the past decade, seven studies have endeavored to measure and report on the release of myokines in response to NMES applied to critically ill patients (50,54,58–62). It is noteworthy that some authors have integrated NMES as part of a multimodal intervention in the context of rehabilitation. For instance, Kayambu (60) and Silva and cols (50) included passive and active motion, coupled with early mobilization such as transfers and/or ambulation, alongside NMES. This combination complicates the isolation of the systemic effects attributable solely to NMES.

Moreover, NMES intervention protocols have exhibited considerable variation across studies. Generally, NMES was administrated with an amplitude ranging from 20-70 mA, employing symmetrical biphasic square waves with a frequency of 30 to 50 Hz. Contraction phases (“On” phase) typically lasted 6-12 seconds, followed by a 1.5 to 2-second increase, a 0.75-second decrease, and a rest period (“Off” phase) lasting 6 to 25 seconds. The pulse duration was consistently set at 400 μs. Current amplitude was often adjusted to elicit “visible contractions in each muscle group” (50,60–62), a criterion that may differ from the “maximal visible contraction” referenced in certain studies, but aligns with the NMES implementation in our protocol.

Regarding the duration of physical intervention, it ranged from 20 minutes (59,63–70) 25 minutes (50), 30 minutes (60,71), and 50-60 minutes (54,58) across the literature, with no apparent differences among them. This rules out the possibility of a dose-effect stemming from longer NMES sessions, thereby supporting the notion that our minimal
changes in myokines are not correlated with session duration, which in our case lasted 30 minutes.

**Myokine selection: true challenge.**

The selection of candidate myokines is particularly challenging due to the vast number biomolecules recognized as myokines (over 600)(43) and the potential impaired capacity from the critically ill patient’s muscle to release some of these in presence of elevated proteasomal pathways (inflammation, atrophy and age contributing to it) (27,36). Even for the physical intervention, referred as any means of muscle activation, heterogeneous results are reported regarding myokine’s plasmatic secretion or gene expression in response to it. Files and cols, considering a one-week early rehabilitation with two physical therapy sessions per day, including passive and resistance training, showed no difference between usual care and an early rehabilitation approach regarding TNF-α, IL-6 and IL-8 levels (72). Franca and cols after one single session of early passive cycle ergometer at lower limbs, reported no significant changes in TNF-α, IFN-g, IL-6 and IL-10 serum levels, comparing before and after intervention (68). And regarding NMES Silva and cols (2019) showed that 25 minutes of NMES applied once a day for 14 days had no impact on TNF-α, IL-1 β; IL-6 and IL-8 plasmatic levels (50).

However, it’s important to note that some studies have reported changes in myokine secretion or gene expression in response to NMES, but sometimes with inconsistent findings. For instance, Block and cols (54), after single-leg NMES twice a day for one hour over one week, observed significantly elevated mRNA expression of both Myostatin and GDF-15 in the NMES-treated legs compared to baseline, while Myostatin levels remained unchanged in the control legs. In contrast, Grunow and cols (59) implemented a comprehensive intervention spanning two weeks, incorporating various muscle activating measures including daily NMES (administered bilaterally on eight different muscle groups for 20 minutes) and/or whole-body vibration (daily for 20 cycles), alongside protocol-based physiotherapy. They noted a significant decrease in
MSTN gene expression in skeletal muscle compared to healthy controls. Additionally, plasma Myostatin levels were found to decrease during the initial 2 weeks of ICU stay (p-value < 0.001), particularly during the early phase of ICU treatment.

This recent evidence, coupled with our own wherein Myostatin was detectable only in only 3 out of 11 patients, suggests that Myostatin may not be a key driver of muscle wasting in critically ill patients (59,73). This raises questions regarding whether this myokine should be retained for further investigations, contrary to our initial expectations based on previous reports (74,75).

Systemic effects of NMES: alternative mechanisms

The positive findings on diaphragmatic function along with the negative findings in the myokines analyzed must prompt us to consider other potential mechanisms mediating the effect of peripheral NMES on respiratory muscles.

Even though NMES was applied to patients under sedation, it could trigger responses such as a withdrawal reflex due to sensory inputs or even mild discomfort, leading to isolated spontaneous breathing efforts. These breathing efforts, in an indirect manner, could promote the preservation of the diaphragmatic structure through spontaneous muscle contraction. Patients were sedated but not unresponsive. The sedation level was generally at Riker scale 2-3 (sedation-agitation scale), indicating that patients can respond to physical stimuli and even move spontaneously. This aligns precisely with what NMES constitutes—a well-defined physical stimulus systematically challenging the patient’s muscles in controlled and closely monitored sessions. Unfortunately, we did not register spontaneous breathing activity during the NMES sessions in this first series of patients. To address this new hypothesis, we plan to incorporate in the near future a continuous airway pressure registration during the NMES sessions to detect spontaneous breathing efforts.

Another plausible mechanism mediating the effect of peripheral NMES on respiratory muscles could be related to the fact that NMES is applied to a mixed peripheral
nerve, typically recruiting both efferent and afferent fibers. This dual recruitment might generate a central feedback loop to respiratory motor cortical areas. In this context, exogenous stimulation could constitute a substitute for endogenous neural drive (76). Cortical areas responsible for voluntary diaphragmatic motion have been identified in the bilateral primary motor areas, adjacent to the regions activating the upper and lower limbs, as recently suggested (77). The brain's respiratory control network must orchestrate muscular synergies that integrate ventilation with posture and body movement, including locomotion of the limbs (78). In a more indirect manner, this could represent a central mechanism that hasn't been well-established yet, through which peripheral muscle activation might influence voluntary respiratory mechanisms.

LIMITATIONS AND STRENGTHS

The main limitation of our study is the small sample size. This impacts the internal validity of our findings, needing a larger sample size for more robust conclusions. Additionally, another design-related limitation is the requirement for a minimum of three days of sedation and mechanical ventilation, which was the most frequent reason for exclusion. However, it is crucial to note that this criterion was established to mitigate confounding factors, such as frequent spontaneous ventilation.

One of the strengths of this study is its design as a randomized clinical trial with a control group. A second highlight is the innovative approach to diaphragmatic function assessment through the Twitch manoeuvre. This technique enables a more precise estimation of diaphragmatic function at a very early stage, when dysfunction is not yet established, and the patient remains under sedation. This approach represents a novel technique in Chile and additionally has been enhanced by the implementation of a closed valve system to measure the tracheal pressure response during occlusion, a technical innovation designed for patients on invasive mechanical ventilation who face the risk of pulmonary de-recruitment in the event of disconnection from the MV. We must also
emphasize the technical capability to capture transient changes in diaphragmatic muscle thickness in response to twitch manoeuvre, which has been poorly described in the literature for patients in the ICU, finding reports mostly from healthy population (79). Our report stands as one of the first to comprehensively address the use of “Twitch thickening fraction” during the magnetic phrenic nerve stimulation, serving as proxy for diaphragmatic function in sedated patients.

CONCLUSION

The preliminary data analysed supports the notion that peripheral NMES can preserve respiratory muscle function. It appears that this effect is not mediated by changes in any of the myokines included in the present study. Therefore, alternative mechanisms should be considered to explain how NMES may favour respiratory muscle preservation. The results observed on peripheral muscle layer thickness are yet unconclusive with the limited sample size analysed. Data from a larger number of patients is required to confirm these preliminary conclusions.

FUTURE ACTIONS

Our primary focus will be to comprehensively characterize changes in respiratory muscle performance during the use of NMES in real-time. This involves capturing respiratory pressure deflections during NMES use and identifying potential factors associated with diaphragmatic activation. Additionally, we plan to conduct a physiological study involving healthy subjects to establish the relationship between NMES, respiratory muscle activation (measured through superficial respiratory muscle oxygen consumption), and transient changes in myokines. This latter setting could
mitigate the significant influence of altered muscular metabolic pathways, as in the critical care environment.

Furthermore, we have outlined plans to assess plasma samples from this study with a proteomic approach to identify possible changes in novel myokines. This secondary analysis will be contingent upon approval from the ethics committee. These multifaceted approach aims to deepen our understanding of the intricate interplay between NMES and myokine responses.
REFERENCES


19. Kim WY, Suh HJ, Hong S, Koh Y, Lim C. Diaphragm dysfunction assessed by


54. SAA Bloch, T Syburrah, U Rosendahl, PR Kemp, MJD Griffiths MP. A paradoxical rise in rectus femoris myostatin (GDF-8) and GDF-15 in response to neuromuscular electrical stimulation in critical care. Thorax. 2014;69(Suppl


**SUPPLEMENTARY MATERIAL**

Table S1. Detailed muscle conformation by day, intervention, and corporal side

<table>
<thead>
<tr>
<th>Patient</th>
<th>Group</th>
<th>Right quadriceps</th>
<th>Left quadriceps</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 1</td>
<td>Day 3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MLT   RF    VI</td>
<td>MLT   RF    VI</td>
</tr>
<tr>
<td>1</td>
<td>C</td>
<td>3.94  1.98  1.94</td>
<td>3.93  1.97  1.96</td>
</tr>
<tr>
<td>2</td>
<td>C</td>
<td>2.06  1.15  0.89</td>
<td>1.19  0.61  0.57</td>
</tr>
<tr>
<td>6</td>
<td>C</td>
<td>1.23  0.55  0.66</td>
<td>1.09  0.54  0.54</td>
</tr>
<tr>
<td>8</td>
<td>C</td>
<td>1.57  0.78  0.74</td>
<td>1.36  0.86  0.51</td>
</tr>
<tr>
<td>9</td>
<td>C</td>
<td>2.81  1.85  0.98</td>
<td>2.29  1.28  1.03</td>
</tr>
<tr>
<td>11</td>
<td>C</td>
<td>1.32  0.74  0.58</td>
<td>1.21  0.7  0.52</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>2.16 ± 1.18 ± 0.9 ±</td>
<td>1.85 ± 0.99 ± 0.86 ±</td>
</tr>
<tr>
<td>3</td>
<td>NMES</td>
<td>2.39  1.38  1</td>
<td>2.29  1.16  1.11</td>
</tr>
<tr>
<td>4</td>
<td>NMES</td>
<td>1.73  0.85  0.86</td>
<td>1.68  1.11  0.57</td>
</tr>
<tr>
<td>5</td>
<td>NMES</td>
<td>1.29  0.64  0.66</td>
<td>1.05  0.46  0.59</td>
</tr>
<tr>
<td>7</td>
<td>NMES</td>
<td>1.65  0.88  0.77</td>
<td>1.44  0.74  0.44</td>
</tr>
<tr>
<td>10</td>
<td>NMES</td>
<td>2.89  1.36  1.53</td>
<td>2.8  1.34  1.46</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>1.9 ± 1.02 ± 0.9 ±</td>
<td>1.85 ± 0.96 ± 0.83 ±</td>
</tr>
</tbody>
</table>

MLT: Muscle Layer Thickness; RF: Rectus Femoris; VI: Vastus Intermedius; C: Control; NMES: Neuromuscular Electrical Stimulation; SD: standard deviation
Figure S1. Change in Twitch tracheal pressure. In red, percentual change for twitch tracheal pressure (Ptr.tw) from day 1 to 3, in the neuromuscular electrical stimulation group (NMES). In black lines, percentual change for Ptr.tw in control group. All values have been normalized according to baseline value.
Figure S2. *twitch tracheal pressure processing*. Green curves represent pressure-time curves. The first positives waves, correspond to passive cycles provided by the ventilator. Following negative curves are the ones induced by the twitch maneuver. In the right panel Δy correspond to the value of $P_{tr, tw}$; calculated graphically from the difference of the lower pressure during twitch (value of negative spike in yellow dotted line), and the pressure value just before twitch (pause pressure in solid yellow line). Curves correspond to assessment of patient number 3 (NMES group), the top image correspond to day 1, and the one in the bottom to day 3.
Table S2. Additional factors related to NMES muscle response

<table>
<thead>
<tr>
<th>Patient</th>
<th>Group</th>
<th>Maximum NMES intensity (mA)</th>
<th>Steroids use</th>
<th>VAD use (Dose)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>NMES</td>
<td>81</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>NMES</td>
<td>40</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>NMES</td>
<td>52</td>
<td>No</td>
<td>NAD (3.75)</td>
</tr>
<tr>
<td>7</td>
<td>NMES</td>
<td>113</td>
<td>No</td>
<td>NAD (1.9)</td>
</tr>
<tr>
<td>10</td>
<td>NMES</td>
<td>56</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

Mean ± SD or Proportion (%) as appropriate: 68.4 ± 29.06 2/5 (20%) 2/5 (20%)

NMES: Neuromuscular electrical stimulation; mA: Mili amperes; VAD: Vasoactive drugs; NAD: Noradrenaline.

Figure S3. Individual myostatin plasmatic concentrations over time. Dots and squares correspond to individual values. NMES group in red symbols. In black control group values. D1: Day 1 ; D3: Day 3; D4: Day 4; T₀: Baseline; T₁: At the end of NMES session; T₂: 2 hours after T₁, and T₆: 6 hours after T₁.
Figure S4. Ethics committee certificate of approval

CERTIFICADO

DR. EMILIANO SOTO ROMO, en calidad de Presidente del Comité Ético-Científico (CEC), del Servicio de Salud Metropolitano Central, constituido por resolución exenta N°1303 de fecha 26 de septiembre del 2002 de la Dirección de dicho Servicio y Acreditado por la SEREMI-RM mediante resolución N° 048975 del 30 de Julio del 2015 y re Acreditado mediante Resolución exenta N° 014561, del 22-12-2021 de la SEREMI-RM, certifica que en sesión expedita del 04 de Febrero del 2022, el CEC-SSMC acusa recibo de carta fechada el 28 de Enero del 2022 y recibida el 02 de Febrero del 2022, el Kinesiólogo Don Yorschua Jalil Contreras, del Departamento Ciencias de la Salud, Escuela de Kinesiología de la Pontificia Universidad Católica de Chile, ubicada en Marquelete 367 Santiago, Región Metropolitana, investigador principal del estudio denominado: “Efecto de la electro estimulación neuroperiférica sobre el perfil de miokineras y la función muscular respiratoria en pacientes críticos. Estudio exploratorio”, a realizarse en la Unidad de Cuidados Intensivos del Hospital Clínico UC Chris tus y donde el rol de subinvestigador lo ejercerá el Kinesiólogo Don Felipe Diamani, del mismo centro asistencial.

En esta oportunidad, el investigador remite para análisis y aprobación los siguientes documentos con los cambios solicitados por el CEC-SSMC en la reunión plenaria del 26 de Enero del 2022:

-Protocolo de investigación versión 2.0, del 02-02-2022. Documento foliado de 12 páginas.

-Documento de Consentimiento Informado versión 3.0, del 03-02-2022. Documento foliado de 06 páginas.

Luego de la presentación de los encargados del análisis preliminar, lectura de los documentos y considerando los criterios relevantes en el análisis de protocolos: utilidad social, validez científica, investigador idóneo, relación riesgo-beneficio favorable, selección equitativa de las personas, protección a la confidencialidad y uso de consentimiento informado, el CEC-SSMC ha decidido aprobar:

-Protocolo de investigación "Efecto de la electro estimulación neuroperiférica sobre el perfil de miokineras y la función muscular respiratoria en pacientes críticos. Estudio exploratorio", versión 2.0, del 02-02-2022. Documento foliado de 12 páginas.

-Documento de Consentimiento Informado versión 3.0, del 03-02-2022. Documento foliado de 06 páginas. Se firma, se fecha y se timbra.

Se recuerda a los investigadores que:

- Una vez aprobado el estudio por parte del CEC-SSMC, el investigador tiene la obligación de informar y solicitar la autorización para llevar a cabo el protocolo de investigación, al Director del establecimiento, quien a su vez definirá la delegación de función de quien lo representará, en la firma del Consentimiento informado correspondiente.
- La validación ética dura un año y de acuerdo a la actual normativa, el investigador tiene la responsabilidad de solicitar la revalidación al término de ese periodo, junto con comunicar al CEC, todo lo relacionado con el estudio: modificaciones, enmiendas, eventos adversos, desviaciones, suspensión del estudio, término del estudio, cierre del sitio, etc.

- Se recuerda, además, que dado que este protocolo será ejecutado con la firma del Consentimiento Informatado hecha por el representante o tutor del paciente mientras el sujeto no recupere su capacidad de consentir, el investigador principal deberá informar a la SEREMI de la Región Metropolitana la validación ética efectuada por este Comité a su proyecto.

- Para los estudios que duren menos de un año, los investigadores tienen el compromiso de hacer llegar el informe de término de la investigación.

- El CEC-SSMC tiene la facultad de realizar visitas en terreno a los sitios de investigación, como parte del seguimiento de los estudios. De acuerdo a la normativa vigente, dichas visitas se avisarán con al menos 48 horas de antelación.

Para ingresar las nuevas versiones de documentos, se solicita a los investigadores hacer llegar:

- Carta conductora dirigida al Dr. Emiliano Soto Romo, solicitando la aprobación, (traer en duplicado).

Se adjunta copia de carta enviada por el investigador, firmada, fechada y timbrada. 
Nº de recepción Nº 52

La sesión expedita, contó con la presencia de la Sra. Carmen Gloria Notario Sánchez, Dr. Rafael Mendizabal Rodríguez y el Dr. Emiliano Soto Romo.
Dr. Emiliano Soto Romo  
Presidente  
Comité Ético Científico del Servicio de Salud M. Central  
CEC-SSMC  

Santiago, 03/02/2022

PRESENTE (4) 04-02-2022 Acta Nº 08

Presentación de nueva versión de Proyecto de Investigación

Estimado Dr. Soto:

En mi calidad de Investigador Responsable (IR) del Proyecto “Efecto de la electroestimulación neuromuscular periférica sobre el perfil de miókinas y la función muscular respiratoria en pacientes críticos. Estudio exploratorio.” A realizarse en la Unidad de Cuidados Intensivos del Hospital Clínico UC Christus, presento a nueva revisión por el Comité Ético Científico del Servicio de Salud M. Central, las versiones 2.0 y 3.0 del protocolo de investigación y documento de consentimiento informado respectivamente, en virtud de los cambios solicitados por el comité tras el pronunciamiento inicial.

Indique lo que corresponda según su protocolo:

- Protocolo investigación versión 2.0
- Documento de Consentimiento Informado versión 3.0 (CI)
- Curriculum Vitae de investigador principal y subinvestigador
- Carta declaración de ausencia de conflicto de interés y apego a las buenas prácticas (si corresponde)
- Carta compromiso
- Carta de aceptación del jefe de servicio

Yorschua Jaeli
Nombre y Firma del Investigador Responsable  
03/02/2021  
Fecha  
04 FEB 2022