"Effect of Physical Therapy on NLRP3 Inflammasome Activation and Muscle Atrophy in Critical Illness Myopathy"

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Abstract
Critical Illness myopathy (CIM) is a common cause of acquired weakness in the intensive care unit. It is defined as a proximal predominant tetra-paresis, usually symmetrical and with respiratory muscle involvement. This condition is a usual complication in intensive care unit (ICU) and has been associated with increased mortality and healthcare costs. Myopathy in the critically ill patient can progress to post-intensive care syndrome, which results in persistent cognitive, mental and physical dysfunction. In Chile, 40% of patients with critical illness due to COVID-19 developed this syndrome. This pathology presents muscle atrophy and alters of contractility, and several cellular mechanisms have been associated with its development, but its etiopathogenesis is still unclear. The role of the ubiquitin proteosome system and atrogenes expression has been linked to its genesis. In murine models of sepsis and denervation, which replicate the characteristics of the critically ill patient, NLRP3 inflammasome complex produces muscle atrophy by overexpression of atrogenes. The NLRP3 inflammasome is a multiprotein complex that plays a pivotal role in inflammatory signalling, and secretion of IL-1β and IL-18. Overexpression of this complex has been reported in CIM, but **is NLRP3 inflammasome activation associated with muscle atrophy due to atrogen upregulation in critically ill patients?** One of the most important risk factors for this myopathy is mechanical silencing, which, unlike other factors, is modifiable by early mobilization. Physical therapy has demonstrated different effects at the cellular level in the skeletal muscle of these patients. **In different pathological conditions, physical therapy is a regulator of inflammation, IL-1β and NLRP3 inflammasome levels, but does physical therapy decrease NLRP3 inflammasome activation in the skeletal muscle of patients with CPM, ¿thereby contributing to a reduction in muscle atrophy?** Based on this background, the following hypothesis is proposed: **Physical therapy decreases NLRP3 inflammasome activity and reduces muscle atrophy in critical illness myopathy.** To test this assertion, in the early stage of ICU stay, patients at risk of this condition (n=16) will be randomized to be treated with conventional physical therapy or to additional physical therapy using servo-assisted motorized movement therapy device, 2 times a day for 60 minutes. In addition, patients without this condition will be considered as controls (n=8). From biopsy samples of the vastus lateralis muscle, histology studies will be performed to determine muscle atrophy and the structure of cellular organelles, expression of atrogenes mRNA by RT-qPCR, protein levels of key mediators involved with NLRP3 inflammasome activation by Western blot, and transcriptomics by microarray. The above to address the following specific aims: a) To evaluate the effect of physical therapy on NLRP3 inflammasome activation in vastus lateralis biopsies from patients with CIM, and to compare this activity with control patients, b) To evaluate the effect of physical therapy on muscle atrophy in vastus lateralis biopsies from patients with CIM, and to relate these results with NLRP3 inflammasome activation, c) To evaluate the effect of physical therapy on the gene expression profile of signalling pathways involved in skeletal muscle atrophy in patients with CIM, and d) To analyze the association between gene expression and skeletal muscle biochemical parameters with the clinical diagnosis of CIM. Extra physical therapy is expected to decrease NLRP3 inflammasome activation, muscle atrophy, and differential expression of genes related to the development of CIM. Additionally, it is expected that these variables are associated with the clinical and ultrasound diagnosis of CIM. **Due to the functional deficit suffered by patients who survive critical illness, it is importance to study the mechanisms underlying to this disorder.** This study proposes a translational approach to achieve this purpose, furthermore, to explore **the contribution of the non-pharmacological intervention for the prevention and management of the critical illness myopathy: the physical therapy.**

1. Background

1.1. Critical illness myopathy

Critical illness myopathy is a common cause of intensive care unit-acquired weakness (ICUAW)(1). ICUAW is a neuropathy and/or myopathy characterized by symmetric, predominantly proximal muscle weakness(2,3), without involvement of facial or ocular muscles(3,4). Respiratory muscles may also be affected(4,5), leading to diaphragmatic dysfunction associated with mechanical ventilation (ventilator-induced diaphragmatic dysfunction, VIDD), which complicates weaning from ventilatory support(6). ICU-AW may be triggered by: a) a neurogenic disorder known as critical illness polyneuropathy (CIP), b) a myogenic disorder referred to as critical illness myopathy (CIM), and/or c) the coexistence of both conditions(4,7–10). CIP is an acute, acquired sensorimotor axonal neuropathy caused by inflammation of the peripheral nerves (6,11). CIM is characterized by impaired muscle mass and contractility[6,12], and presents with pathognomonic signs such as preserved sensory function, elevated creatine kinase levels(11), and most notably, a preferential loss of myosin compared to actin in muscle biopsy samples(6,12,13). Since muscle atrophy and impaired contractility are key features associated with increased mortality(14–18) and physical disability(19,20), much of the research has focused on CIM and the molecular pathways involved in skeletal muscle degradation(21).

The development of critical illness myopathy (CIM) is associated with the severity of the critical illness(22–24). Both modifiable and non-modifiable risk factors have been identified(3,4,25). Modifiable factors include hyperglycaemia(8,25–28), parenteral nutrition(4,8,25,27,29), use of vasopressor agents(4,25,27), neuromuscular blockade(4,8,10,11,25,27,28,30,31), corticosteroid therapy(2,4,8,11,25,27,31), prolonged sedation and analgesia(4,25,31), use of aminoglycoside antibiotics(4,25,26,28), prolonged mechanical ventilation(2,8,25–27,29,31), extended ICU stay(8,29), and mechanical silencing due to prolonged immobilization(4,25,27–29,31). Non-modifiable risk factors include specific diagnoses and life-support measures associated with critical illness, such as sepsis(25–27,29,32), systemic inflammatory response syndrome (SIRS)(4,8,25,32), multiple organ failure (MOF)(2,4,25–27,32), hyperlactatemia(4), and renal replacement therapy(26). Patient-related characteristics such as age(4,25,29), comorbidities(4), and female sex (4,25,27) have also been identified as non-modifiable contributors.

Regarding the diagnosis of CIM, different strategies are employed depending on whether the patient is cooperative or non-cooperative. Another key aspect is the differential diagnosis between CIM and CIP. In awake and cooperative patients, muscle strength is clinically assessed using the Medical Research Council Sum Score (MRC-SS)(3,9,33). A score of 48 or below indicates muscle weakness, with scores below 36 denoting severe weakness(4,33,34). Handgrip dynamometry is another diagnostic tool in conscious patients; values below 11 kg for men and 7 kg for women are indicative of CIM. However, the validity of handgrip strength as a representative measure of global muscle strength has been questioned(35). Electrophysiological evaluation is also used to diagnose CIM and can be applied in unconscious or non-cooperative patients, as well as for differential diagnosis between CIP and CIM(34,36). Nerve and muscle biopsies may provide valuable mechanistic insight and enable differential diagnosis; however, due to their invasive nature, biopsies are recommended only within a research context(33,34). In non-cooperative patients, various imaging techniques have been employed to assess muscle mass as a surrogate for muscle strength. Computed tomography (CT) and magnetic resonance imaging (MRI) allow for the evaluation of muscle quality, detection of fatty infiltration, and quantification of lean muscle mass(16); Nevertheless, these methods are costly and require specialized personnel and software(16,34). Among imaging tools, quadriceps muscle ultrasound appears the most promising, as it permits rapid, bedside, and repeatable assessments of both quantitative parameters (muscle thickness, anatomical and physiological cross-sectional area) and qualitative features (echogenicity and muscle architecture)(18,34,37). Diagnosis of CIM by muscle ultrasound is based on changes in sonographic variables observed within 7 to 10 days of critical illness progression, including a ≥20% reduction in muscle thickness, ≥10% decrease in cross-sectional area, ≥5% decrease in pennation angle, and an 8–10% increase in echogenicity(17,18,37). Early diagnosis is of critical importance, as protein degradation, muscle mass loss (38,39), and electrophysiological

alterations(40) occur within the first 10 days of ICU admission. iven the relevance of early detection, several critical illness severity scores—such as the Sequential Organ Failure Assessment (SOFA) and the Acute Physiology and Chronic Health Evaluation II (APACHE II)—have been proposed. When combined with muscle ultrasound assessment, these tools may be sufficient to raise suspicion and support the clinical consideration of this condition (22,23,41,42).

CIM is a common complication among critically ill patients(17,31,43) and is associated with increased mortality(9,11,25,28,29,31), prolonged hospital stays(9,10,25,28), longer durations of mechanical ventilation(10,29), higher healthcare costs, and reduced long-term physical function and quality of life(9,10,29,31,44). Moreover, this condition may progress to post-intensive care syndrome (PICS), which can result in persistent cognitive, mental, and physical dysfunction(4,28,45). A multicentre Chilean study evaluating the functional and cognitive sequelae of PICS in patients who experienced critical illness due to COVID-19 found that 40% of survivors exhibited some degree of disability after hospital discharge(46). Therefore, understanding the cellular and molecular etiopathogenesis of CIM is essential for developing both pharmacological and non-pharmacological therapeutic strategies.

1.2.Potential Mechanisms and Pathogenesis of CIM

CIM is characterized by reduced muscle mass and impaired contractility(44,47–49). In vivo muscle biopsies from critically ill patients, postmortem tissue samples, and animal models of CIM have revealed skeletal muscle fiber atrophy and degeneration(21,50–52). Biopsies from patients with CIM suggest that these alterations result from an imbalance between protein synthesis and degradation, the latter being exacerbated through: a) activation of the ubiquitin-proteasome system (UPS), b) cytosolic proteases such as calpains and caspases, and c) dysregulation of autophagy pathways. Additional mechanisms described include decreased muscle excitability, impaired neuromuscular transmission, channelopathies, altered calcium homeostasis, mitochondrial dysfunction, and oxidative stress, among others(4,33,38,44,53). Although multiple etiological mechanisms have been proposed in the pathophysiology of CIM, its precise aetiology and causative pathways remain unclear. Therefore, further biomedical research is needed to elucidate the pathogenesis of CIM and to develop novel preventive and therapeutic strategies.

1.3.Muscle Atrophy, Inflammation, and the NLRP3 Inflammasome

The primary risk factors for the loss of muscle mass and strength, hallmarks of CIM, include sepsis, SIRS, and elevated levels of inflammatory markers(54). Among the proinflammatory cytokines involved in the development of muscle atrophy are tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 (IL-1)(55). In addition to systemic inflammation, skeletal muscle is now recognized as a secretory organ capable of releasing various myokines (peptides, metabolites, cytokines, among others), including IL-6 and IL-1β.(55). The processes of gene expression, cleavage, and secretion of IL-1β are tightly regulated(56). Since IL-1β is translated as an inactive precursor (pro-IL-1β), its cleavage is mediated by the NLRP3 inflammasome (55,56), a multiprotein complex essential to inflammatory signalling (57). This complex is composed of the NLRP3 receptor protein, the ASC adaptor protein, and pro-caspase-1 as the effector component(57). In addition to its role as a key regulator of muscle metabolism(58,59), the NLRP3 inflammasome has been implicated in various myopathies(60–62), the development of sarcopenia(63,64) and sepsis-induced muscle atrophy in C2C12 myotubes(65). In patients with CIM, two transcriptomic analyses have reported differential expression of the NLRP3 gene(66) and functional enrichment of the Gene Ontology category “NLRP3 inflammasome complex”(67) in muscle biopsies from the tibialis anterior and vastus lateralis, respectively. In murine models of denervation and sepsis, NLRP3-associated muscle atrophy appears to be mediated by the upregulation of the E3 ubiquitin ligases MuRF1 and Atrogin-1, with NLRP3 gene deletion conferring a protective effect(68,69). The upregulation of these ligases is well documented in human CIM(12,40,42,66,70–73), and in various experimental models of this myopathy(74–82). Additionally, increased expression of other skeletal muscle E3 ligases, such as MUSA1(66,73), TRIM62(73), and TRIM32(66), has been described. This upregulation is associated with enhanced proteasome activity in muscle samples from critically ill patients with muscle atrophy.(42,71,83). Taken together, evidence

from murine models of denervation and sepsis indicates that the NLRP3 inflammasome contributes to muscle atrophy via upregulation of the UPS(68,69). Since these models replicate key features of CIM and increased NLRP3 gene expression has been reported in patients with CIM, it is plausible to propose that the NLRP3 inflammasome contributes to muscle atrophy in human CIM.

1.4.NLRP3 Inflammasome Activation in CIM: Priming and Oligomerization

Canonical activation of the NLRP3 inflammasome occurs in two steps. The first signal, known as "priming," is triggered by the detection of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) via Toll-like receptors (TLRs), or through the activation of tumor necrosis factor-alpha (TNF-α) and interleukin-1β (IL-1β) receptors(57). This in turn activates the nuclear factor kappa B (NF-κB), which upregulates the transcription of NLRP3, pro-IL-1β, and pro-IL-18(57,84,85).

Given that the NLRP3 inflammasome can be activated by various inflammatory and pathogen-associated stimuli(85,86), and that sepsis and SIRS are common risk factors for CIM(4,25–27,29,32,87), the critical illness environment provides favorable conditions for priming. Supporting this notion, increased expression of TNF-α, TNFR, and IL-1β has been reported in the muscle tissue of patients with CIM(40,66,71,73,88), as well as in murine models of sepsis (89).

Moreover, overexpression of genes related to the NF-κB signaling pathway has been documented in skeletal muscle from patients with CIM(90) and in a murine ICU model(77), suggesting a potential pivotal role of this transcription factor in CIM pathogenesis. Therefore, it is reasonable to hypothesize that priming of the NLRP3 inflammasome may occur during the development of CIM.

Regarding the second activation signal, or the assembly of the NLRP3 inflammasome complex, this also occurs in response to PAMPs and DAMPs(57,84,85). Oligomerization of the NLRP3 protein leads to the cleavage of pro-caspase-1, generating active caspase-1(57,85). This enzyme subsequently processes pro-IL-1β and pro-IL-18 into their active forms. Caspase-1 also cleaves gasdermin D (GSDMD), and the N-terminal fragment (GSDMD-NT) oligomerizes to form pores in the plasma membrane, facilitating the secretion of IL-1β and IL-18 and initiating inflammatory responses, including pyroptotic cell death(57,84,85,91).

A broad array of intracellular conditions and mediators contribute to this second activation step(57,84,85). Among them, mitochondrial dysfunction and the production of reactive oxygen species (ROS) are considered major stimuli for NLRP3 inflammasome activation(92–94). Mitochondrial dysfunction is well documented in critical illness and during the early stages of CIM(38). This has been evidenced by: (a) disruption of mitochondrial ultrastructure(89,95–99); (b) dysregulation of mitochondrial biogenesis factors—such as TFAM(95,98–100), TFBM1, TFBM2(98–100), PGC1α(42,88,95,96,98,100–103), PGC1β(100), PPARγ2(82), PPRC1(102), NRF-1(95,98,100), and NRF-2(98,100,101); (c) increased mitophagy as indicated by elevated Bnip3 expression(40,70,72,76,98,104); (d) reduced ATP synthesis and oxidative phosphorylation(88,89,105,106); (e) alterations in levels, expression, and activity of mitochondrial respiratory complexes(79,80,95–97,99–101,103,106–108); (f) redox imbalance reflected by changes in superoxide dismutase levels and reductions in various antioxidant enzymes(51,95,99,100,103,104); and (g) increased oxidative stress, as shown by lipid peroxidation, protein carbonylation, and nitration(70,97,100,103,109). Taken together, these findings support the hypothesis that activation and assembly of the NLRP3 inflammasome may occur in human CIM. Additionally, the release of lysosomal contents such as cathepsin B also contributes to this second signal and NLRP3 inflammasome assembly(85,110). In CIM, increased expression and activity of cathepsin L(40,70,72) and cathepsin B(66,72), among others(66), have been reported, along with an increased presence of lysosomal vacuoles and vesicles(13,70). Therefore, mitochondrial dysfunction, oxidative stress, and elevated cathepsin activity—features described in CIM—may be mechanistically linked to the second activation signal of the NLRP3 inflammasome. However, this association has not yet been specifically studied in CIM.

1.5. Physical Therapy, Inflammation, and Potential Modulation of the NLRP3 Inflammasome in CIM

Mechanical silencing due to prolonged bed rest is a key risk factor in the development of CIM. Importantly, this factor is modifiable through early mobilization(4,25,27–29,31). **However, further evidence is needed to support the notion that physical therapy**(3,111)**, active mobilization, and rehabilitation in critically ill patients can improve muscle strength and functional status. These interventions may reduce the incidence of CIM and shorten hospital stays**(3,4,25,112–116).

Physical therapy has demonstrated biological effects on the muscles of critically ill patients, as well as in experimental models of CIM. In a murine ICU model, physical therapy was shown to exert a protective effect against myosin loss, while increasing the expression of sarcomeric proteins and growth factors involved in muscle development(80). Kalamgi et al. reported that physical therapy increased levels of Akt, p-Akt, p-S6, as well as the expression of myogenin and MyoD(76), suggesting that mobilization promotes protein synthesis and muscle repair pathways in murine models of critical illness. In a randomized controlled trial, Hickmann et al. described a reduction in REDD1 expression(40), which may enhance mTOR signaling and protein synthesis(117). The same study reported decreased expression of Bnip3, Gabarapl1, and Lc3b, and increased p-ULK1^{Ser757}, a condition that inhibits autophagy. Additionally, the authors observed reduced colocalization of p62 with Lamp-2A(40), suggesting a decrease in chaperone-mediated autophagy. In a murine ICU model, mechanical loading was found to induce PGC-1α4 (an exercise-induced isoform)(76), thereby promoting mitochondrial biogenesis. Based on current evidence, physical therapy and early mobilization in critically ill patients may exert effects on mitochondrial function and autophagy, potentially influencing NLRP3 inflammasome activation. **However, this regulatory role has not yet been demonstrated in the skeletal muscle of critically ill patients with CIM.** Regarding physical therapy and its relationship with systemic inflammation, several studies have described its modulatory effect on the inflammatory state across different health conditions(118–132). arious forms of exercise and physical therapy have been shown to reduce levels of IL-1β(124,131,132) andTNF-α(124,128,130) in multiple pathologies. In elderly populations, exercise reduces levels of NLRP3 and caspase-1(133). Furthermore, physical therapy and exercise have been associated with reduced NLRP3 inflammasome activity in murine models of Parkinson's disease(134); post-stroke depressio(135); and chronic kidney disease–associated cachexia(136) Similarly, in a murine model of sciatic nerve injury, decreased levels of IL-1β and TNF-α were observed(137). However, the potential modulatory effect of physical therapy on the NLRP3 inflammasome and its role in the development of CIM has not yet been described. The pathogenesis of inflammation-associated muscle atrophy remains unclear (84), particularly in CIM. Therefore, understanding the molecular mechanisms underlying this condition is of great importance.

In summary: (1) Skeletal muscle from patients with CIM shows overexpression of the NLRP3 inflammasome gene and associated signaling components; (2) the NLRP3 inflammasome promotes muscle atrophy through upregulation of atrogenes in murine models of sepsis, denervation, and in C2C12 myotubes; (3) critically ill patients exhibit systemic inflammation, sepsis, and elevated levels of TNF-α and IL-1β—conditions that promote NLRP3 inflammasome priming; (4) CIM is characterized by mitochondrial dysfunction, oxidative stress, and increased cathepsin B, all of which favor the second activation signal of the NLRP3 inflammasome; (5) in various pathological contexts, physical therapy has been shown to reduce inflammation, IL-1β, TNF-α, and NLRP3 inflammasome levels, suggesting it may act as a protective factor against NLRP3-mediated muscle atrophy in CIM.

Based on these observations, the following research questions arise: In human CIM: (a) Is NLRP3 inflammasome activation associated with muscle atrophy via upregulation of atrogenes?; (b) Does physical therapy reduce NLRP3 inflammasome activation in the skeletal muscle of patients with CIM, thereby contributing to the attenuation of muscle atrophy?

Figure 1 provides a graphical summary of the proposed contribution of the NLRP3 inflammasome to muscle atrophy in CIM, and the potential protective effect of physical therapy.

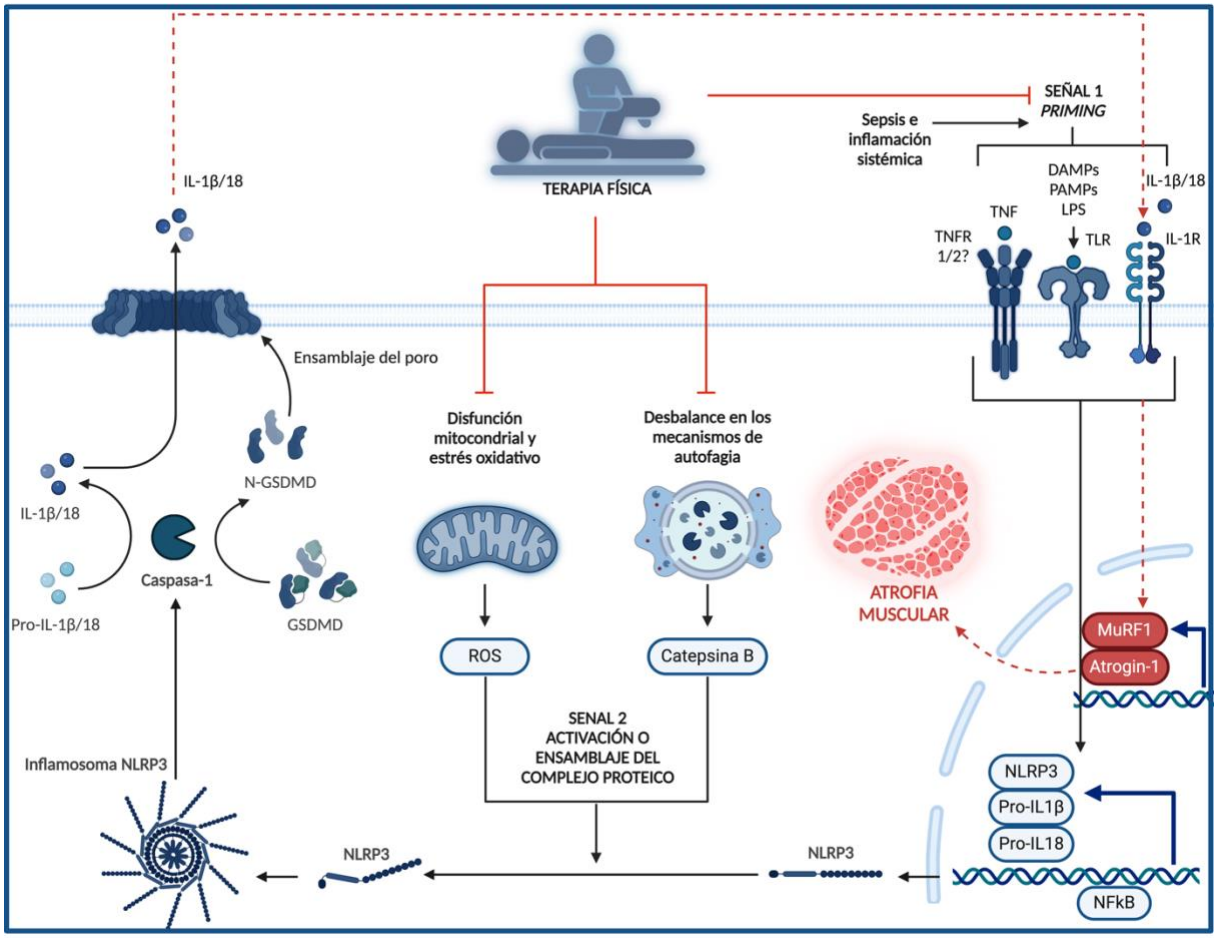

Figure 1. Proposed contribution of the NLRP3 inflammasome to muscle atrophy in critical illness myopathy and the protective effect of physical therapy.

Based on the aforementioned background, the hypothesis of this research project is:

2. Hypothesis

Physical therapy reduces NLRP3 inflammasome activity and mitigates muscle atrophy in critical illness myopathy.

3. General Objective

To investigate the association between the effects of physical therapy on NLRP3 inflammasome activation and muscle atrophy in critical illness myopathy.

4. Specific Objectives

4.1. SO1: To evaluate the effect of physical therapy on NLRP3 inflammasome activation in vastus lateralis biopsies from patients with CIM, and to compare this activity with that of control patients.

4.2. SO2: To assess the effect of physical therapy on muscle atrophy in vastus lateralis biopsies from CIM patients, and to relate these findings to NLRP3 inflammasome activation.

4.3. SO3: To determine the effect of physical therapy on the gene expression profile of signaling pathways involved in skeletal muscle atrophy in CIM patients.

4.4. SO4: To analyze the association between gene expression and skeletal muscle biochemical parameters and the clinical diagnosis of CIM.

5. Experimental Design

SO1: To evaluate the effect of physical therapy on NLRP3 inflammasome activation in vastus lateralis biopsies from CIM patients, and to compare this activity with that of control patients.

Rationale: The NLRP3 inflammasome causes muscle atrophy in murine models of CIM (68,69). Prolonged bed rest, a major risk factor for CIM, is modifiable through physical therapy (4,25,27–29,31). This intervention has been shown to modulate inflammation(118–132) and the NLRP3 inflammasome(133), reduce levels of IL-1β and TNF-α(124,128), and influence mitochondrial function and autophagy(40), potentially leading to decreased inflammasome activation. However, this relationship has not been explored in human CIM.
Experimental approach: Patients with CIM will be randomized to receive conventional or additional physical therapy. Non-CIM patients will serve as controls. From serum and vastus lateralis biopsies, the following will be assessed:

1. **NLRP3 inflammasome priming:** Protein levels of phospho-p65^{S536} and total p65 will be measured via Western blot; mRNA levels of NLRP3, IL-1β, and IL-18 via RT-qPCR.
2. **NLRP3 inflammasome activity:** Cleaved IL-1β, cleaved caspase-1, and GSDMD-NT will be quantified by Western blot relative to their precursor forms. IL-1β and IL-18 levels in plasma will be measured by ELISA.
3. **Second-signal activation factors:** Oxidative stress markers (protein carbonylation and nitration) and cathepsin B levels will be evaluated by Western blot and RT-qPCR. In addition, mitochondrial ultrastructure and the presence and volume of lysosomal vacuoles will be analysed by transmission electron microscopy as a strategy to assess mitochondrial dysfunction and dysregulated autophagy.

Expected outcomes: Physical therapy is expected to reduce NLRP3 inflammasome activation in a frequency- and duration-dependent manner.
Pitfalls: If difficulties arise in measuring the proposed signaling pathway from muscle biopsy lysates, primary skeletal muscle cultures will be used to study inflammasome activity. Caspase-1 activity will also be assessed using a fluorometric kit (FAM-FLICA™, Bio-Rad).

SO2: To assess the effect of physical therapy on muscle atrophy in vastus lateralis biopsies from CIM patients, and to relate these findings to NLRP3 inflammasome activation.

Rationale: Muscle mass loss in CIM is partly attributed to atrogene overexpression(7). In murine models, NLRP3 contributes to atrophy through this mechanism (68,69). However, this association—and its modulation by physical therapy—has not yet been explored in humans.
Experimental approach: The characteristics of CIM and muscle atrophy will be correlated with findings from SO1:

1. **Muscle atrophy:** Muscle cryosections (10 µm) will be analyzed by indirect immunofluorescence using anti-laminin to delineate cell boundaries and quantify fiber diameter. mRNA levels of atrogenes—MuRF1, Atrogin-1, MUSA1, TRIM62, and TRIM32—will be measured by RT-qPCR.
2. **Molecular and histological features of CIM:** Myosin/actin ratio will be quantified by Western blot using biopsy homogenates. Sarcomeric organization will be evaluated in 10 µm cryosections using transmission electron microscopy.

Expected outcomes: Physical therapy is expected to reduce muscle atrophy in relation to intervention frequency and duration. These effects are expected to be associated with changes in NLRP3 inflammasome activation.
Pitfalls: If the proposed evaluations are not feasible, miRNA expression related to muscle atrophy will be assessed via nanopore sequencing. If immunofluorescence is not possible, myofiber cross-sectional area will be analyzed using H&E histology.

SO3: To determine the effect of physical therapy on the gene expression profile of signaling pathways involved in skeletal muscle atrophy in CIM patients.
Rationale: Transcriptomics has advanced rapidly in critical care, enabling early diagnosis, precision medicine(138,139), and identification of key signaling pathways associated with gene expression changes (138,140). Such analyses have been applied in CIM patients(66,67,141),

and murine models(76,80,142). However, only one study has investigated the effect of physical therapy on transcriptomics in CIM, and it did not show significant effects, likely due to its sample size and unilateral intervention(66). More studies are needed to uncover the underlying mechanisms of CIM and the impact of early mobilization.
Experimental approach: Differential gene expression related to NLRP3 inflammasome and skeletal muscle atrophy pathways will be assessed via microarray.
Expected outcomes: Greater differential expression of genes associated with muscle atrophy is expected in CIM patients. Physical therapy is expected to modulate this expression.
Pitfalls: If technical or interpretative issues arise with the microarray, mRNA sequencing will be performed using nanopore technology.

SO4: To analyze the association between gene expression and skeletal muscle biochemical parameters with the clinical diagnosis of CIM.
Rationale: Early diagnosis of CIM is crucial. Although tools like the SOFA score and muscle ultrasound have shown promise(39,40), more evidence is needed to validate their use(41).
Experimental approach: Molecular biology findings and gene expression profiles will be associated with the clinical and ultrasound-based diagnosis of CIM, in conjunction with critical illness severity, to support early detection strategies.
Expected outcomes: A strong association is expected between early CIM diagnosis, clinical markers, and biochemical/histological findings.

6. Patients and Methods

6.1.Study Design

This pilot study adopts a quantitative approach, with an analytical, experimental, prospective, and longitudinal design.

6.2.Patients

6.2.1. CIM Patients (Group A, n = 16)

Inclusión Criteria: Critically ill patients with CIM will be selected based on the following inclusion criteria:

1. Medical diagnosis of sepsis upon ICU admission,
2. Receiving invasive mechanical ventilation with a projected requirement ≥7 days,
3. SOFA score ≥8 for three consecutive days within the first five days of ICU admission (22,41,42,81,141,144).

To screen for CIM in these patients, serial quadriceps ultrasound assessments will be conducted at three timepoints: within the first 24 hours of ICU admission, again within the first 72 hours, and finally 7 days after the second assessment. The ultrasound protocol is detailed in Annex 2. Changes in muscle thickness, cross-sectional area, pennation angle, and echogenicity will be evaluated. Patients meeting the following criteria within 7–10 days of ICU admission will be considered suspected CIM cases: ≥20% reduction in muscle thickness, ≥10% reduction in cross-sectional area, ≥5% decrease in pennation angle, ≥8% increase in echogenicity(37).

For patients who regain consciousness and are cooperative, muscle strength will be assessed using the Medical Research Council Sum Score (MRC-SS); a score ≤48 confirms muscle weakness(9,33,145).

Exclusion criteria: Patients will be excluded if they present with: neurocritical illness, prior malnutrition or cachexia, pre-existing neuromuscular disease, coagulopathy (severe liver disease or continuous dialysis), thrombocytopenia <20,000 platelets/μL, inability to walk independently prior to hospitalization or low pre-admission functional status (Clinical Frailty Scale ≥4), lower limb amputation, lower limb fractures, ongoing chemotherapy, pregnancy, BMI >35, uncontrolled epilepsy, allergy to ultrasound gel, prolonged corticosteroid therapy, expected ICU stay <7 days, imminent death, or legal guardian refusal to provide informed consent.

Group A Subgroups:

- **A.1**: CIM patients receiving conventional physical therapy
- **A.2**: CIM patients receiving additional physical therapy (servo-assisted lower limb mobilization using Motomed Letto® at 30 rpm, 2 sessions/day, 60 minutes/session for 7 days).

Although no specific treatment exists for CIM(66), prior studies have evaluated the effect of prolonged physical therapy compared to standard care (40,66), allowing all patients access to this intervention regardless of standardization(40,147,148). Therapy dosage may be titrated by adjusting rpm intensity, under safety criteria defined by Hodgson's expert consensus(149) and continuous monitoring of hemodynamic and respiratory parameters.

6.2.2. Control Patients (Group B, n = 8)

Control patients will be recruited from those admitted for conditions unrelated to CIM and who are conscious and capable of providing informed consent.

Exclusion criteria: Same as for Group A, with the addition of: laboratory evidence of systemic inflammation (elevated C-reactive protein and/or leukocytosis) and refusal to consent.

6.2.3. Patient Recruitment Sites

All Group A and B patients will be recruited from the Adult Critical Care Unit at the Hospital de Urgencia y Asistencia Pública (Ex Posta Central) and Clínica INDISA. Each participant will have a clinical file where all morbidity data will be recorded. Either the patient or their legal guardian will sign an informed consent form outlining all study procedures. All information will be treated with strict confidentiality.

6.3. Sample Size Validation

The sample size was calculated based on the increased gene expression of IL-1β observed in vastus lateralis muscle biopsies from critically ill patients with sepsis, reported as 15.36 times higher than that of healthy controls (with a standard deviation of 4.96), according to Hickmann et al.(40). This expression level is proposed as the effect size, or the variable to be reduced by physical therapy, under the assumption that IL-1β gene expression reflects NLRP3 inflammasome activation in skeletal muscle.

Assuming a statistical power of 99%, a type I error (alpha) of 5%, and an anticipated 40% mortality rate in critically ill patients (to account for potential loss)(150), the estimated sample size was 8 patients with CIM per physical therapy group. Based on the principle of equivalence, the control group will also include 8 patients. Sample size was calculated using R Studio with the following command: **n.ttest(power = 0.99, alpha = 0.05, mean.diff = 15.36, sd1 = 4.96, design = "unpaired", variance = "unequal").**

6.4.Sample Collection from Patients with CIM and Controls

6.4.1. Vastus Lateralis Muscle Biopsy

Two samples of the vastus lateralis muscle will be obtained from both CIM patients and controls: the first within the initial 3 days of ICU admission, and the second 7 days later. The muscle biopsy procedure is well-established in the literature (12,13,39,40,42,49,67,71–73,81–83,88,95,99,101,104–106,141,144,151). A percutaneous needle biopsy (TPNB) will be used, as it is a minimally invasive technique(152) that has demonstrated good yield in critically ill patients for gene expression, protein, and ultrastructural analyses(40). Under local anesthesia with 2% lidocaine, three to five samples of the vastus lateralis (VL) muscle will be collected using a disposable 12-gauge coaxial biopsy needle (BARD, MAGNUM®) to obtain approximately 90 mg of tissue. The first sample will be taken at a 90° angle and stored at -80°C for histological studies. The remaining samples will be cleared of blood with sterile gauze, snap-frozen in liquid nitrogen, and stored at -80°C for gene and protein expression analysis.

6.4.2. Blood Samples

Each CIM patient and control subject will donate 10 ml of peripheral blood on two occasions, collected in EDTA vacutainers (Becton Dickinson Ind Cir Ltda.) as anticoagulant. The sampling will be synchronized with the timing of muscle biopsy collection.

6.5.Evaluators

The methodology requires ultrasound assessment of the quadriceps muscle and the use of the MRC Sum Score and FSS-ICU scales by physical therapists from the ICU. Although these professionals have clinical experience with these evaluations and adequate inter-rater reliability has been reported in the literature(145,153–158), it is necessary to verify inter-evaluator concordance. A prior pilot study titled *“Validation of Operators and Inter-Evaluator Variability in Diagnostic Strategies for ICU-Acquired Weakness”* will be conducted in cooperative patients capable of providing consent. This study is under development and has received ethical approval from the Scientific Ethics Committee of Clínica INDISA (Protocol No. 176-25-24).

6.6. Histological and Biochemical Methods

6.6.1. Histology

Transmission electron microscopy (TEM) will be performed on ultrathin 10 µm cryosections from muscle biopsy fragments. Sections will be fixed in 2.5% glutaraldehyde for 2 hours at room temperature, washed four times in 0.1 M sodium cacodylate buffer, dehydrated in graded acetone series, and embedded in Epon resin. Ultrathin sections will be contrasted sequentially with 2% osmium tetroxide in 0.1 M sodium cacodylate buffer and 1% uranyl acetate in the same buffer, then examined via TEM.

6.6.2. Immunofluorescence

10 µm cryosections will be washed with TBS-T (Tris-buffered saline with 0.1% Tween20®), fixed with 100% cold acetone for 30 minutes at -20°C, then blocked with 3% BSA in TBS-T for 2 hours. Permeabilization will be performed using 0.1% Triton X-100 in 3% BSA for 30 minutes. Primary antibody against laminin (1:30, L9393, Sigma-Aldrich) will be incubated overnight at 4°C, followed by secondary antibody Alexa Fluor 488 Goat anti-Rabbit (1:500, Life Technologies) for 2 hours at room temperature in the dark. Slides will be mounted with ProLong containing DAPI (Life Technologies), stored at 4°C, and imaged by confocal microscopy. Muscle fiber diameter will be measured using ImageJ software and compared against control values to determine muscle atrophy.

6.6.3. Western Blot

30 mg of muscle biopsy samples will be lysed using an automated homogenizer (D1000, Biolab, Madrid, Spain) in a lysis buffer containing: 1X T-PER (#78510, ThermoFisher, Massachusetts, USA), 1 mM EGTA (Winkler, Santiago, Chile), 1 mM EDTA (Winkler, Santiago, Chile), 2 mM BAPTA (#A4926, Sigma Aldrich, Missouri, USA), 10 mM $NaVO_4$ (#S6508, Sigma Aldrich, Missouri, USA), 10 mM $Na_2P_2O_7$ (Winkler, Santiago, Chile), 1X protease inhibitor (#04693124001, Roche, Mannheim, Germany) and 1X phosphatase inhibitor (#04906845001, Roche, Mannheim, Germany). Samples will then be centrifuged at 4°C for 14 minutes at 12,000 rpm. The supernatant will be analyzed by SDS-PAGE (8% or 10%, depending on the target protein). Wet electrotransfer will be performed onto polyvinylidene fluoride (PVDF) membranes, which will be blocked with 5% semi-skimmed milk (SVELTY®) in TRIS-buffered saline with 0.1% Tween 20 (TBS-T), followed by TBS-T washes. Membranes will be incubated with the primary antibody for 16 hours at 4°C. Subsequently, incubation with the appropriate HRP-conjugated secondary antibody will be carried out—either anti-rabbit (1:10,000, 7074, Cell Signaling) or anti-mouse (1:10,000, 516102, Santa Cruz Biotechnology). Membranes will be developed using enhanced chemiluminescence (ECL) reagent (sc-2048, Santa Cruz Biotechnology), and images will be acquired with the LI-COR C-Digit imaging system and Image Studio software (Lincoln, Nebraska, USA). Individual protein levels and protein-to-protein ratios will be compared with those obtained from control patients without neuromusculoskeletal disease.

6.6.3.1. Myosin/Actin Ratio

Membranes will be incubated with two antibodies: anti-myosin antibody diluted 1:1000 (F59: sc-32732, Santa Cruz Biotechnology), and anti-actin antibody diluted 1:1000 (HUC1-1: sc-53141, Santa Cruz Biotechnology). The myosin/actin ratio will then be calculated. A ratio below 1.5 is considered conclusive for myopathy (6,12,13,90).

6.6.3.2. Priming of the NLRP3 Inflammasome

To assess the phosphorylation levels of p65 (a component of the NF-κB complex), membranes will be incubated with two antibodies: anti-p65S536 antibody diluted 1:1000 (sc-101752, Santa Cruz Biotechnology) and total anti-p65 antibody diluted 1:1000 (sc-8008, Santa Cruz Biotechnology).

6.6.3.3. Activation of the NLRP3 Inflammasome

To evaluate the activation of the NLRP3 inflammasome, the ratios of cleaved caspase-1/pro-caspase-1, cleaved IL-1β/pro-IL-1β, and GSDMD-N/GSDMD will be determined.

6.6.3.3.1. Cleaved Caspase-1/Pro-Caspase-1 Ratio

Membranes will be incubated with two antibodies: anti-cleaved caspase-1 antibody diluted 1:1000 (4199, Cell Signaling) and anti-caspase-1 antibody diluted 1:1000 (3866, Cell Signaling).

6.6.3.3.2. Cleaved IL-1β/Pro-IL-1β Ratio

Membranes will be incubated with two antibodies: anti-cleaved IL-1β antibody diluted 1:1000 (83186, Cell Signaling) and total anti-IL-1β antibody diluted 1:1000 (12703, Cell Signaling).

6.6.3.3.3. GSDMD-N/GSDMD Ratio

Membranes will be incubated with two antibodies: anti-GSDMD-N antibody diluted 1:1000 (ab215203, Abcam) and anti-GSDMD antibody diluted 1:400 ((H-11): sc-393581, Santa Cruz Biotechnology).

6.6.3.4. Secondary Activation of the NLRP3 Inflammasome

To assess conditions that favor the secondary activation of the NLRP3 inflammasome, oxidative stress and increased cathepsin B due to dysregulated autophagy will be evaluated. Oxidative stress will be assessed by measuring protein carbonylation and nitration. Protein carbonylation will be detected using the OxyBlot™ Protein Oxidation Detection Kit (EMD Millipore), according to the manufacturer's instructions. Protein nitration will be assessed using anti-nitrotyrosine antibody diluted 1:2000 ((39B6): sc-32757, Santa Cruz Biotechnology). Cathepsin B levels will be quantified using anti-cathepsin B antibody diluted 1:1000 (H-5: sc-365558, Santa Cruz Biotechnology). GAPDH will be used as a loading control, detected with anti-GAPDH antibody diluted 1:2000 ((0411): sc-47724, Santa Cruz Biotechnology).

6.6.4. RT-qPCR

A total of 20 mg of muscle biopsy tissue will be lysed using an automatic homogenizer (D1000, Biolab, Madrid, Spain) in 1 mL of TRIzol reagent (Invitrogen). The lysate will be incubated at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes. Then, 200 µL of chloroform per mL of TRIzol will be added to the homogenate tubes and vigorously shaken for 30 seconds. After 2–3 minutes of incubation, the samples will be centrifuged at 12,000 g for 15 minutes at 4°C. The aqueous phase will be transferred to a new microcentrifuge tube. To precipitate RNA, 200 µL of isopropanol per 1 mL of TRIzol used will be added and incubated for 2 hours at -20°C. The samples will then be centrifuged at 12,000 g for 10 minutes at 4°C, and the supernatant will be discarded. For RNA washing, the pellet will be suspended in 1 mL of 75% ethanol at -20°C (same volume of TRIzol used for lysis), briefly vortexed, and centrifuged at 7500 g for 5 minutes at 4°C. After discarding the supernatant, the RNA pellet will be air-dried for 5–10 minutes. To solubilize the RNA, the pellet will be resuspended in 20–30 µL of RNase-free water and incubated on a heat block at 55–60°C for 10–15 minutes. RNA concentration will be measured using the MaestroNano spectrophotometer.

Reverse transcription will be performed using the iScript cDNA Synthesis Kit (Bio-Rad). Reactions will be carried out in a final volume of 10 µL containing 1 µg of RNA, 0.1 µL of each primer (100 nM final), and 5 µL of cDNA. Thermal cycling will follow this protocol: 3 minutes at 95°C, followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. SYBR® Green (Applied Biosystems) will be used as the fluorescent probe. All samples will be run in duplicate. β2-microglobulin (β2M) will be used as the reference gene, as it has shown stable expression levels regardless of the exercise modality (159).

6.6.4.1. Priming of the NLRP3 Inflammasome

To assess the priming of the NLRP3 inflammasome, RT-qPCR will be performed to quantify mRNA levels of NLRP3, IL-1β, and IL-18.

6.6.4.2. Secondary Activation of the NLRP3 Inflammasome

To evaluate dysregulated autophagy favoring the secondary activation of the NLRP3 inflammasome, RT-qPCR will be used to assess Cathepsin B mRNA expression.

6.6.4.3. Atrogenes

To evaluate the ubiquitin-proteasome system, RT-qPCR will be performed to quantify mRNA levels of the ubiquitin ligases: MurF1, Atrogin-1, MUSA1, TRIM62, and TRIM32.

Primer List:

Gene	Forward Primer	Reverse Primer
NLRP3	5'-GAGGAAAAGGAAGGCCGACA-3'	5'-CCCGGCAAAAACTGGAAGTG-3'
IL-1β	5'AGCAACAAGTGGTGTTCTCCATGT-3'	5'-AGCTGTAGAGTGGGCTTATCATCTT-3'
IL-18	5'-AGCCTAGAGGTATGGCTGTAAC-3'	5'-AAAGGTCTCTCTCTTTTTCACAAGC-3'
Catepsina B	5'-GCGCTGGGTGGATCTAGGA-3'	5'-GTTGACCAGCTCATCCGACA-3'
MurF1	5'-AAACAGGAGTGCTCCAGTCGG-3'	5'-CGCCACCAGCATGGAGATACA-3'
Atrogina-1	5'-CGACCTCAGCAGTTACTGCAA-3'	5'-TTTGCTATCAGCTCCAACAG-3'
MUSA1	5'-GTGATCATGCCAATCCACAG-3'	5'-CATGAATGTCACCATGCACA-3'
TRIM62	5'-AAGTCCCTGTTCCAGGACATC-3'	5'-GCAGTCGTCCGACAGGAT-3'
TRIM32	5'-CCCCATAGGGGATGAGAAAT-3'	5'-GTCCTATTTGGGCAGTGCAT-3'
Fbxo21/SMART	5'-CACGTTCCTTGTAATGGCTTCA-3'	5'-ACGACTCATAGTCATCTGGCTG)-3'
β2M (Housekeeping)	5'-ATGAGTATGCCTGCCGTGTGA-3'	5'GGCATCTTCAAACCTCCATG-3'

6.6.5. ELISA

EDTA vacutainer tubes will be centrifuged for 10 minutes at 4°C and 4000 rpm. Plasma and serum will be aliquoted into microcentrifuge tubes with 500 µL per sample. Measurements will be performed according to the manufacturer's instructions using the following kits: IL-1β: Human IL-1β ELISA Kit (Invitrogen), IL-18: Human IL-18 ELISA Kit (Invitrogen).

6.6.6. Microarray.

Total mRNA will be extracted from samples of control and CIM (Critical Illness Myopathy) patients. Using reverse transcriptase (RT), complementary DNA (cDNA) libraries will be synthesized from the extracted RNA. These cDNA libraries will be labeled with fluorescent probes incorporated during synthesis. The labeled cDNA samples will then be hybridized to a commercial microarray chip containing oligonucleotides with complementary sequences. This hybridization process results in a stable DNA double helix. After hybridization, the chip will be washed several times to remove any unbound cDNA. The chip will then be scanned using a laser to excite the fluorescent tags. The fluorescence signal from each spot will be captured and analyzed with specialized software that generates a colorimetric image of the microarray. Expression profiles will be obtained using Illumina HT-12 v4 microarrays (one microarray per sample).

6.6.6.1. Microarray Data Analysis

Raw expression data will be processed using the R software environment and the "Bioconductor" package. The data will undergo background correction, quantile normalization, and log2 transformation using the neqc function from the *limma* package. Probes classified as "No match" or "Bad" will be removed using the *illuminaHumanv4* annotation package.

6.6.6.2. Differential Gene Expression

Robust differential expression analysis of all probes will be performed using the *limma* package in R, adjusted for each patient's sociodemographic and clinical variables. Sample correlation will be assessed using the duplicateCorrelation function. The decideTests function with the "global" adjustment will be used to estimate the false discovery rate (FDR), using a significance threshold of FDR < 0.05 and a minimum expression change of 1.5-fold compared to controls.

6.6.6.3. Gene Ontology (GO)

Functional enrichment analysis in Gene Ontology will be performed using the "gProfiler" tool in R. The statistical threshold for GO enrichment will be an FDR-adjusted p-value < 0.05, with a minimum of 10 detected genes. The enrichment list will include all genes represented in the Illumina Human HT-12 v4 array with a p-value < 0.05 in at least three samples.

6.7. Demographic Variables, Severity of Critical Illness, Clinical Assessment of Muscle Strength, and Functionality

The sociodemographic and clinical variables that will be recorded throughout the study include: biological sex, age, body mass index (BMI), degree of hypoxemia, comorbidities, route of nutrition, nitrogen balance, serum albumin, lactatemia, duration of mechanical ventilation, length of ICU stay, and total hospitalization. The severity of critical illness will be assessed using the SOFA (Sequential Organ Failure Assessment) score. The use and dosage of the following medications will also be recorded: norepinephrine, milrinone, dobutamine, propofol,

ketamine, midazolam, dexmedetomidine, neuromuscular blocking agents, corticosteroids, and aminoglycosides.
Regarding physical therapy and functionality, the Clinical Frailty Scale(160) will be used to determine the functional status prior to ICU admission. In cooperative patients (those who respond to at least 3 out of 5 commands in the S5Q scale—five standardized ICU questions), analytical muscle strength will be assessed using the Medical Research Council Sum Score (MRC-SS)(9,33,145). A score of ≤48 points confirm the presence of muscular weakness, which must be corroborated by ultrasound and SOFA scale findings. This scale also allows for the classification of severe muscle weakness when the score is below 36 points(33,34,41). Functional status will be assessed using the Functional Status Score for the ICU (FSS-ICU)(46,158,161). Data collection will include the time elapsed until the first physical therapy session, the number of sessions, the modality of intervention, and its temporal distribution.

6.8.Statistical Analysis
Primary quantification results will be expressed as gene or protein expression levels relative to control patients. These will be reported using central tendency and dispersion measures, and normality will be assessed using the Shapiro–Wilk test.
For comparisons between independent groups, Student's t-test or the Wilcoxon rank-sum test will be applied, depending on the distribution of the data. To assess differences before and after the early physical therapy intervention, paired Student's t-tests or Wilcoxon signed-rank tests will be used.
Correlation between the outcome variables of Objective 1 (SO1) and Objective 2 (SO2) will be analyzed using Pearson or Spearman correlation coefficients, depending on variable distribution.
For differential gene expression analysis, quantification will be relative to control patients, and significance will be assessed with a false discovery rate (FDR) threshold of <0.05.
Associations between variables will be analyzed based on their nature. Multiple linear regression will be applied to quantitative variables. For associations between cellular and molecular biology findings and dichotomous variables (e.g., presence of muscle weakness), logistic regression will be used. For associations with ordinal variables, such as functional scales (e.g., FSS-ICU), Poisson regression will be applied. All statistical analyses and graph generation will be conducted using R Studio and Stata software.

7. Ethical Considerations

7.1.Social Utility

Critical illness myopathy (CIM) is a common cause of ICU-acquired weakness (ICUAW)(1). This condition is characterized by muscle atrophy and impaired contractility, both of which contribute to increased mortality(14–18), prolonged hospital stays (9,10,25,28), (extended durations of mechanical ventilation(19,20), elevated healthcare costs(9,10,29,31,44), and long-term physical disability(19,20). CIM may progress to post-intensive care syndrome (PICS), potentially resulting in persistent disability(4,28). A Chilean study reported that 40% of critically ill patients experience some degree of disability after hospital discharge(46). Therefore, understanding the etiology of CIM is essential for designing both pharmacological and non-pharmacological therapeutic strategies.

Several risk factors have been identified in the development of CIM(4,25), with prolonged immobility highlighted as a modifiable factor through physical therapy (4,25,27–29,31). This underscores the importance of evaluating the effects of physical therapy and supports the biomedical justification for dedicated clinical staff to manage and implement rehabilitation in critically ill patients. Since muscle protein degradation and electrophysiological abnormalities emerge within the first 10 days of ICU admission(39,40), early and timely rehabilitation is crucial. Early detection strategies for CIM are necessary. Internationally, research groups have implemented methods combining the Sequential Organ Failure Assessment (SOFA) score with ultrasound assessment to detect CIM in non-cooperative patients(22–24,41,42). While this method has proven effective in specialized research groups, it remains to be broadly adopted in Chile. Implementing this evaluation locally could facilitate early CIM detection and allow for better allocation of physical therapy resources, minimizing mechanical silencing — a modifiable risk factor. Currently, critical care units in Chile do not have full coverage for rehabilitation needs(162). Thus, biomedical research on CIM could benefit the general population by informing public policies that expand access to physical therapy and rehabilitation in intensive care units.

7.2.Protection of Vulnerable Groups

This study focuses on critically ill patients during the first days of admission to the intensive care unit (ICU), a population considered vulnerable due to the lack of consciousness resulting from sedoanalgesia. However, since the study specifically targets the development of critical illness myopathy (CIM), it can only be conducted in this population. The initial hospitalization period is when the risk factors associated with CIM development manifest (22,26,87,163). In accordance with Article 28 of Chilean Law No. 20.584, this study will include adult patients who "are physically or mentally incapable of expressing consent." It is important to note that “this physical or mental condition that prevents providing informed consent or expressing preference is a necessary characteristic of the target research population.”

As such, the study protocol will be submitted for review by a scientific ethics committee. If approved, it will subsequently be submitted for evaluation to the Regional Health Authority (SEREMI). Once approved by these institutions, legal guardians will be approached to provide informed consent for the inclusion of patients in the research protocol. Furthermore, as stipulated by Article 28 of Law No. 20.584, once the patients have recovered from sedation, are cooperative, and demonstrate no delirium or physical incapacity, “consent or expression of preference will be sought from the individuals who have regained the physical or mental capacity to provide such consent or to express their preferences.”

This research does not introduce any form of bias related to sex, race, or age among participants.

7.3.Randomization

Patients at risk of developing CIM (Group A) who meet the inclusion criteria and do not fulfill any exclusion criteria will be randomly assigned to receive either conventional physical therapy or an enhanced physical therapy protocol using a servo-assisted lower limb cycling device (Motomed Letto®). This strategy has been employed in previous studies to assess the effects of physical therapy by comparing extended interventions with conventional protocols(40,66,146–148,164). This design ensures that all patients have access to early

physical therapy and rehabilitation, thereby avoiding comparison with a non-intervention group(40,147).
Randomization will be independent of ethnicity. However, in order to ensure homogeneity across intervention groups, stratification will be performed based on two factors: age and sex. Stratified randomization will be implemented, and allocation concealment will be applied only at the assignment stage. Due to the nature of the intervention—physical therapy—it will not be possible to blind either participants or investigators to the treatment received; thus, the study is classified as open-label.
Randomization will be conducted using R Studio software and the "randomizr" package. Stratification by age and sex will be performed using the "split" function.

7.4.Adverse Events
This study involves performing a muscle puncture to obtain tissue samples from the vastus lateralis. This procedure is well established in the study of CIM (12,13,39,40,42,49,67,71–73,81–83,88,95,99,101,104–106,141,144,151). Although the most commonly used techniques for human muscle biopsy are the percutaneous needle biopsy (Bergström method)(49,67,70–72,82,83,99,101,106,151), conchotome(13,39,66), and Henckel-Tilley forceps(95,105), a more recently described method—tiny percutaneous needle biopsy (TPNB)—has shown advantages. This technique is less traumatic for patients(152) and has already been applied in CIM studies, providing adequate yield for gene expression, protein analysis, and ultrastructural studies without reports of adverse events(40).
The biopsy will be performed under local cutaneous anesthesia (2% lidocaine hydrochloride) with prior disinfection using topical chlorhexidine, employing a sterile technique and a coaxial disposable biopsy needle (BARD, MAGNUM®, C.R. Bard Inc., Covington, GA). To minimize the occurrence of adverse events, biopsies will be performed by intensivist physicians using ultrasound guidance, allowing image-assisted needle placement(165). In parallel, the clinical nurse of the intensive care unit, will supervise and provide nursing care for the biopsy sites.
As previously mentioned, patients at risk of CIM will be randomized to receive either conventional physical therapy or more frequent and prolonged sessions using a servo-assisted lower-limb cycling device (Motomed Letto®). Recent studies have demonstrated the safety of this type of intervention during physiotherapy in intensive care settings(147).
Ultrasound evaluation is routine in intensive care units(166). However, some cases of contact dermatitis due to ultrasound gel have been reported (167–172). Therefore, the skin of the area assessed by ultrasound will be monitored after each session. Additionally, the exclusion criteria in this protocol are designed to minimize the likelihood of adverse events.

7.5.Benefit Assessment
Percutaneous muscle biopsy is recognized as an essential technique for advancing knowledge in muscle physiology and for the diagnosis of musculoskeletal disorders(165,173,174). Although general concerns exist regarding potential adverse events associated with this procedure, the incidence of complications is low and typically of minimal clinical relevance. The most commonly reported complications include pain, erythema, ecchymosis, edema, and bleeding(173). This study entails the inherent risks associated with muscle puncture; however, these are generally mild and self-limiting(173). The likelihood of significant complications is further reduced through the application of strict exclusion criteria.
In the event of a complication arising from the muscle biopsy procedure, medical professional liability insurance will be available to cover any associated harm. It is important to note that participants will not receive any direct clinical benefit from undergoing the biopsy procedure. This will be explicitly explained during the informed consent process to the legal guardians of the patients, and subsequently to the patients themselves, once they regain the cognitive capacity necessary to either continue with or withdraw from the study.
Physical therapy within the ICU is a standard intervention in critically ill patients and will be conducted in accordance with established safety criteria described in the literature(149). Additionally, an enhanced physical therapy regimen will be applied using servo-assisted cycling devices(147,148). Importantly, all enrolled patients will receive some form of physical therapy—either conventional or enhanced—thus ensuring that no participant is deprived of therapeutic benefit.

7.6. Protection of Privacy

All collected data will be securely stored under the custody of the principal investigator. Participant information will be recorded using the REDCap (Research Electronic Data Capture) platform. The presentation of study results will always preserve participant anonymity; no personal identifiers or sensitive data will be disclosed to third parties. Muscle biopsy samples will be processed at the Muscle Metabolism Laboratory, where they will remain under the custodianship of Dr. Paola Llanos Vidal.

7.7. Ethical Monitoring of the Project

Any issues that arise during the implementation of the study or any amendments to the protocol will be reported to the ethics committee. In accordance with Article 10 of Law 20.120, *"Any serious adverse event occurring during the course of the research must be reported by the investigator to the Committee and to the Director of the institution where the research is being conducted."*

7.8. Feasibility

This study is supported by the clinical teams of the Adult Intensive Care Units at the Hospital de Urgencia y Asistencia Pública (Ex Posta Central) and Clínica INDISA, which will serve as the recruitment centers from 2025 through the end of 2026.

The research project is led by Dr. Lilian Jara Sosa, Dr. Paola Llanos Vidal, and Dr. Denisse Valladares Ide. Dr. Lilian Jara Sosa is a geneticist with extensive experience in the field, including research on the genetic profile of myopathies in the Chilean population. Under her guidance, transcriptomic analyses and differential gene expression studies will be conducted using advanced sequencing and bioinformatics technologies.

Dr. Paola Llanos is a specialist in the activation pathway of the NLRP3 inflammasome in skeletal muscle in preclinical models of insulin resistance induced by obesity. She leads investigations focused on low-grade inflammation in skeletal muscle, employing biochemical, cellular, fluorescence and confocal microscopy techniques, as well as muscle fiber cultures.

Dr. Denisse Valladares Ide has extensive expertise in studying the molecular mechanisms underlying sarcopenia using human muscle biopsy samples. She is an active researcher on the modulatory role of physical activity on sarcopenia in elderly populations.

Experiments related to the quantification of markers of NLRP3 inflammasome "priming" and assembly, as well as transcriptomic analyses, will be carried out with funding from Fondecyt Regular Grant No. 1231103 awarded to Dr. Paola Llanos Vidal and Fondecyt Regular Grant No. 1241959 awarded to Dr. Denisse Valladares. The acquisition of the biopsy device and disposable 12-Gauge coaxial biopsy needles (BARD, MAGNUM®) will be financed through the operating expenses of the National Doctoral Scholarship ANID No. 21231808.

Additionally, the project has the formal approval of medical leadership and rehabilitation coordinators of the Adult Intensive Care Units at the Hospital de Urgencia y Asistencia Pública and Clínica INDISA.

The project also includes the participation of Dr. Margot Navarrete, physician, general surgeon and intensive care medicine resident, and Dr. Valentín López, physician and internal medicine specialist, who will be responsible for performing the muscle biopsies.

Clinical nurse Valentina Burgos will assist with the monitoring of the muscle biopsy puncture site and associated nursing care.

The complementary expertise of the thesis advisors, along with a multidisciplinary team, will allow the project to be developed with a translational (bench-to-bedside) approach.

8. Discussion

Why is it important to study Critical Illness Myopathy? CIM is a common condition among critically ill patients(17,31,43), associated with increased mortality (9,11,25,28,29,31), prolonged hospital stay(9,10,25,28), elevated healthcare costs, and reduced physical function and long-term quality of life(9,10,29,31,44). It may also lead to Post-Intensive Care Syndrome (PICS), characterized by persistent cognitive, mental, and physical dysfunction(4,28).

In this study, an increase in NLRP3 inflammasome activation is expected in critically ill patients, potentially contributing to muscle atrophy. NLRP3 inflammasome activation has been linked to the upregulation of muscle atrophy-related genes in established murine models of the disease(68,69). However, this relationship has not yet been described in human CIM. We hypothesize an association between changes in inflammasome activation and muscle atrophy in patients with CIM. This connection would be highly relevant, as muscle atrophy and impaired contractility are key features driving poor outcomes in these patients(14–20). While associations do not necessarily imply causation(3), the findings of this study, derived from ex vivo samples, may support a link between the proposed mechanism and CIM pathogenesis. This could lead to the identification of novel pharmacological and non-pharmacological therapeutic targets, including physical therapy.

Mechanical silencing due to prolonged bed rest is a major modifiable risk factor for the development of CIM(4,25,27–29,31). Physical therapy is a non-pharmacological intervention that could regulate the proposed mechanism of atrophy. This intervention has shown beneficial effects in both CIM patients and experimental models, particularly in improving mitochondrial function(40,76) and autophagy processes(40). Additionally, various modalities of exercise and physical therapy have been shown to reduce IL-1β and TNF-α levels in different pathological conditions(124,126,128,130–132,137). Therefore, physical therapy in critically ill patients could modulate NLRP3 inflammasome activation(57,84,85,91), and the associated muscle atrophy. However, to date, research groups studying CIM pathophysiology have not yet addressed the potential protective effects of physical therapy on this pathway.

As an additional methodological approach to evaluate the mechanisms involved in CIM development and the effects of physical therapy, a genetic screening strategy is proposed. Transcriptomic analyses have previously been performed in both CIM patients(66,67,82) and in murine models of this condition(76,80,142). Nevertheless, only one study has analyzed the impact of physical therapy in patients with CIM, reporting no significant changes in the skeletal muscle gene expression profile(66). It should be noted that this study included only 7 patients and compared one limb with the contralateral immobilized limb(66). In contrast, the present study proposes to assess the effects of conventional physical therapy versus additional physical therapy, as previously described in the literature(40,147). Given the scarcity of such studies and the rationale outlined in this project, there is a clear need to expand knowledge regarding the effects of early mobilization and the underlying mechanisms of CIM.

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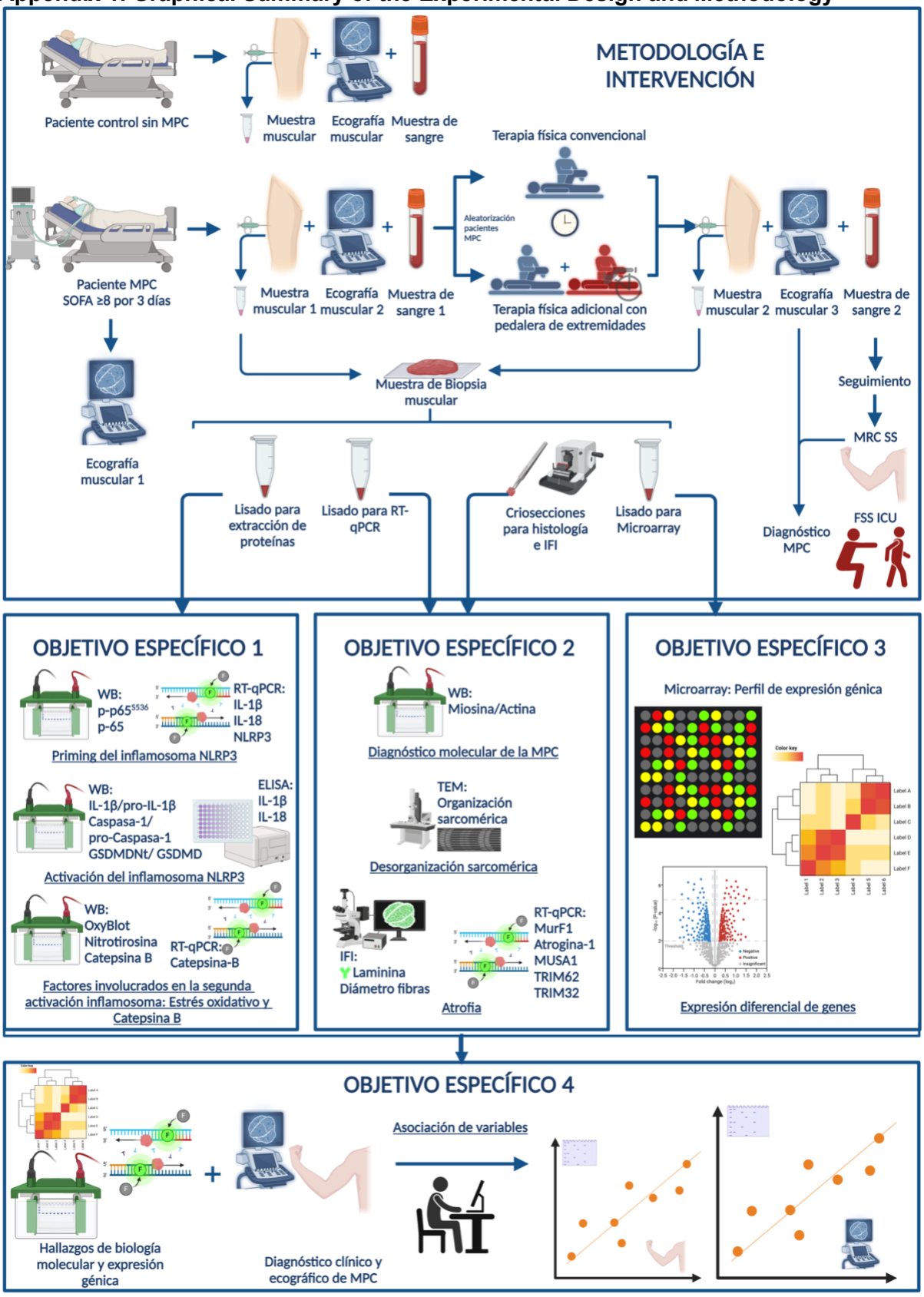
10. Gantt Chart

	2024		2025				2026			
ACTIVIDAD	3°	4°	1°	2°	3°	4°	1°	2°	3°	4°
OE1: Evaluar el efecto de la terapia física en la activación del inflamosoma NLRP3 en biopsias del vasto lateral de pacientes con MPC, y comparar esta actividad con la de pacientes control.										
Priming del inflamosoma NLRP3 - NLRP3, IL-1β e IL-18 mRNA - RT-qPCR				■	■	■	■	■	■	
Priming del inflamosoma NLRP3 - p65S536/p65t - Western blot				■	■	■	■	■	■	
Actividad del inflamosoma NLRP3 - Caspasa-1/pro-caspasa-1 IL-1β/pro-IL-1β GSDMD/NGSDMD - Western blot				■	■	■	■	■	■	
Actividad del inflamosoma NLRP3. IL-1β - IL-18 - ELISA				■	■	■	■	■	■	
Fact. ~ con ensamblaje I. NLRP3 - Estrés oxidativo - Dinitrofenol, Nitrotirosina - Western Blot				■	■	■	■	■	■	
Fact. ~ con ensamblaje I. NLRP3 - Catepsina B - RT-qPCR/Western Blot				■	■	■	■	■	■	
OE2: Evaluar el efecto de la terapia física sobre la atrofia muscular en biopsias de vasto lateral de pacientes con MPC, y relacionar estos resultados con la activación del inflamosoma NLRP3.										
Diagnóstico molecular de la MPC - Razón Miosina/Actina - Western blot				■	■	■	■	■	■	
Diagnóstico molecular de la MPC - Desorganización sarcomética - TEM				■	■	■	■	■	■	
Atrofia muscular - IFI laminina				■	■	■	■	■	■	
Atrogenes- MurF1, atrogina-1, MUSA1, TRIM62 y TRIM32 mRNA - RT-qPCR				■	■	■	■	■	■	
OE3: Evaluar el efecto de la terapia física en el perfil de expresión génica de las vías de señalización involucradas en la atrofia del músculo esquelético en pacientes con MPC.										
Ensayo de transcriptómica por Microarray				■	■	■	■			
Análisis estadístico y bioinformático: DEG, GO							■	■	■	
OE4: Analizar la asociación entre la expresión génica y los parámetros bioquímicos del músculo esquelético con el diagnóstico clínico de la MPC.										
Análisis estadístico de la asociación entre los hallazgos bioquímicas del músculo y el diagnóstico clínico de la MPC.								■	■	
Otros										
Estandarización técnica: WB - RT-qPCR - Histología e IFI	■	■	■							
Proyecto piloto Variabilidad inter-evaluador: MRC SS, FSS ICU, ecografía muscular y ecografía de nervio periférico		■	■							
Examen de calificación		■								
Presentación proyecto a Comité de Ética			■							
Presentación proyecto a SEREMI de Salud			■							
Registro proyecto en ClinicalTrials				■						
Escritura artículo científico - Protocolo				■						
Diseño e incorporación plataforma REDCap				■						
Avance 1						■				
Avance 2									■	
Escritura artículo científico									■	■
Escritura de tesis									■	■
Tesis final										■

Appendix 1. Graphical Summary of the Experimental Design and Methodology



Appendix 2. Ultrasound Evaluation Procedure

1. Muscle Ultrasound

Ultrasound assessments of the quadriceps muscle will be performed within the first 72 hours of ICU admission (Day 1) and again at Day 7. To identify the site for the transverse measurement, the patient will be placed in a supine position with the knee joint in a neutral resting position. A line will be drawn between the anterior superior iliac spine (ASIS) and the superior pole of the patella, and measurements will be taken at the junction between the middle and lower third of this line. At this measurement point, the thickness of the anterior quadriceps complex—including the rectus femoris (RF) and the vastus intermedius (VI)—as well as its cross-sectional area (CSA), will be assessed. Images will be acquired for echogenicity analysis using ImageJ software.
For longitudinal measurements, a point 5 cm lateral to the transverse measurement site will be marked, where the pennation angle will be evaluated. A linear transducer with a frequency range of 4 to 12 MHz (Sonosite™