Mechanisms Underlying Intensive Care Unit Muscle Wasting

*Intervention Strategies in an Experimental Animal Model and in Intensive Care Unit Patients*

MONICA LLANO-DIEZ
Dissertation presented at Uppsala University to be publicly examined in Hedstrandsalen, Akademiska sjukhuset, Ingång 70, by, Uppsala, Thursday, June 14, 2012 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Critically ill patients admitted to the intensive care unit (ICU) commonly develop severe muscle wasting and weakness and consequently impaired muscle function. This not only delays respirator weaning and ICU discharge, but has deleterious effects on morbidity, mortality, financial costs, and quality of life of survivors. Acute Quadriplegic Myopathy (AQM) is one of the most common neuromuscular disorders underlying ICU muscle wasting and paralysis, and is a consequence of modern intensive care interventions, although the exact causes remain unclear. Muscle gene/protein expression, intracellular signalling, post-translational modifications, muscle membrane excitability, and contractile properties at the single muscle fibre level were explored in order to unravel the mechanisms underlying the muscle wasting and weakness associated with AQM and how this can be counteracted by specific intervention strategies. A unique experimental rat ICU model was used to address the mechanistic and therapeutic aspects of this condition, allowing time-resolved studies for a period of two weeks. Subsequently, the findings obtained from this model were translated into a clinical study. The obtained results showed that the mechanical silencing of skeletal muscle, i.e., absence of external strain (weight bearing) and internal strain (myosin-actin activation) due to the pharmacological paralysis or sedation associated with the ICU intervention, is likely to be the primary mechanism triggering the preferential myosin loss and muscle wasting, features specifically characteristic of AQM. Moreover, mechanical silencing induces a specific gene expression pattern as well as post-translational modifications in the motor domain of myosin that may be critical for both function and for triggering proteolysis. The higher nNOS expression found in the ICU patients and its cytoplasmic dislocation are indicated as a probable mechanism underlying these highly specific modifications. This work also demonstrated that passive mechanical loading is able to attenuate the oxidative stress associated with the mechanical silencing and induces positive effects on muscle function, i.e., alleviates the loss of force-generating capacity that underlie the ICU intervention, supporting the importance of early physical therapy in immobilized, sedated, and mechanically ventilated ICU patients.

Keywords: acute quadriplegic myopathy, intensive care unit, myosin, regulation of contraction, muscle atrophy, mechanical loading, mobilization

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ISSN 1651-6206
ISBN 978-91-554-8387-6
urn:nbn:se:uu.diva-173466 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-173466)
To Víctor
“It is hard to convey just how debilitated one is after an insult of ICU magnitude.”

Professor Cheryl J. Misak

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


Llano-Diez M, Gustafson AM, Olsson C, Goransson H, Larsson L.

III  **Sparing of muscle mass and function by passive loading in an experimental intensive care unit model.** *Manuscript*

IV  **Intensive care unit muscle wasting: mechanisms and intervention strategies.** *Submitted*

* Contributed equally to this study.

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Out of the thesis

doi:10.1371/journal.pone.0020558
Ochala J, Renaud G*, Llano-Diez M*, Banduseela VC, Aare S, Ahlbeck K, Radell PJ, Eriksson LI, Larsson L.

* Contributed equally to this study.
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Abbreviations

ACh  Acetylcholine
ActRIIB  Activin receptor IIB
ALK4/5  Activin receptor-like kinase 4/5
ALS  Autophagy lysosome system
AMP  Adenosine 5´-monophosphate
AMPK  AMP-activated protein kinase
Ankrd2  ankyrin repeat domain 2
ARDS  Acute respiratory distress syndrome
BMPs  Bone morphogenic proteins
CIM  Critical illness myopathy
CIP  Critical illness polyneuropathy
CK  Creatine kinase
CMAP  Compound muscle action potential
CS  Corticosteroids
CSA  Cross-sectional area
DGC  Dystrophin-glycoprotein complex
DHPR  Dyhydropyridine receptor
DMS  Direct muscle stimulation
EDL  Extensor digitorum longus
4eBP1  Eukaryotic initiation factor 4E binding protein 1
eIF2b  Eukaryotic initiation factor 2b
ELC  Essential light chain
EMG  Electromyography
EMS  Electrical muscle stimulation
ER  Endoplasmic reticulum
ERAD  Endoplasmic reticulum-associated degradation
FGF  Fibroblast growth factor
FoxO  Forkhead box O
FST  Follistatin
FSTL-3  Follistatin-like 3
GH  Growth Hormone
GSH  Glutathione
GSK3β  Glycogen synthase kinase 3 beta
HGF  Hepatocyte growth factor
HMM  Heavy meromyosin
HSP  Heat shock protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>i.v.</td>
<td>Intravenously</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive care unit</td>
</tr>
<tr>
<td>IGF-1</td>
<td>Insulin Growth Factor-1</td>
</tr>
<tr>
<td>IKK</td>
<td>Inhibitor of κB kinase</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin receptor substrate</td>
</tr>
<tr>
<td>LMM</td>
<td>Light meromyosin</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MEF2</td>
<td>Myocyte enhancer factor 2</td>
</tr>
<tr>
<td>Mrfs</td>
<td>Myogenic regulatory factors</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>MUP</td>
<td>Motor unit potential</td>
</tr>
<tr>
<td>MuRF</td>
<td>Muscle-specific RING finger protein</td>
</tr>
<tr>
<td>MyBP</td>
<td>Myosin binding protein</td>
</tr>
<tr>
<td>MyHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>MyLC</td>
<td>Myosin light chain</td>
</tr>
<tr>
<td>NMB</td>
<td>Neuromuscular blockade</td>
</tr>
<tr>
<td>NMBA</td>
<td>Neuromuscular blocking agents</td>
</tr>
<tr>
<td>nNOS</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>N-WASP</td>
<td>Neuronal Wiscott-Aldrich syndrome protein</td>
</tr>
<tr>
<td>PGC1α</td>
<td>Peroxisome proliferator activated receptor γ coactivator 1α</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol triphosphate</td>
</tr>
<tr>
<td>PTMs</td>
<td>Post-translational modifications</td>
</tr>
<tr>
<td>QoL</td>
<td>Quality of life</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RyR</td>
<td>Ryanodine receptor</td>
</tr>
<tr>
<td>SF</td>
<td>Specific force</td>
</tr>
<tr>
<td>Shh</td>
<td>Sonic hedgehog</td>
</tr>
<tr>
<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
</tr>
<tr>
<td>SNAP</td>
<td>Sensory nerve action potential</td>
</tr>
<tr>
<td>SR</td>
<td>Sarcoplasmic reticulum</td>
</tr>
<tr>
<td>SRF</td>
<td>Serum response factor</td>
</tr>
<tr>
<td>ST</td>
<td>Specific tension</td>
</tr>
<tr>
<td>STAT3</td>
<td>Signal transducer and activator of transcription-3</td>
</tr>
<tr>
<td>TA</td>
<td>Tibialis anterior</td>
</tr>
<tr>
<td>TGFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Tumour necrosis factor beta</td>
</tr>
<tr>
<td>Tn</td>
<td>Troponin</td>
</tr>
<tr>
<td>TRiC</td>
<td>TCP-1 ring complex</td>
</tr>
<tr>
<td>TWEAK</td>
<td>TNF-like weak inducer of apoptosis</td>
</tr>
<tr>
<td>UPS</td>
<td>Ubiquitin proteasome system</td>
</tr>
</tbody>
</table>
Introduction

Overview of Skeletal Muscle
Skeletal muscle is the largest tissue in the body, comprising 40-45% of the total body weight. Skeletal muscle mass is approximately composed of 70% water, 20% proteins, and 5% substances such as enzymes, minerals, fats, amino acids, carbohydrates and phosphates. In the human body there are approximately 660 skeletal muscles and in physiological terms they can be divided into peripheral muscles (involved in limb movements) and respiratory muscles (involved in breathing). Skeletal muscle is under control of the somatic nervous system and, therefore, is responsible for voluntary movements. In addition to its function to generate force and movement, it is also an important amino acid pool. Amino acids can be released into the circulation and converted to glucose if the liver needs them, i.e., during starvation and critical illness. In addition, skeletal muscle forms a mechanical protection of bone and viscera and it is an important producer of cytokines and growth factors.

Development
Embryonic skeletal muscles (except craniofacial muscles) come from segmented blocks of the paraxial mesoderm called the somites. Muscle progenitor cells delaminate from the four edges of the dorsal part of the somite, the dermomyotome, and later they migrate into the limb buds. There, muscle precursor cells proliferate to myoblast that are mononucleated cells. After differentiation myoblast cannot divide by mitosis and they fuse to form myotubes that eventually will mature into muscle fibres. Several growth and transcription factors and myogenic regulatory factors (Mrfs) regulate this process called myogenesis.

The tyrosine kinase receptor, c-met, together with its ligand, hepatocyte growth factor (HGF), and the transcription factors Pax3 and Lbx1 are key players in delamination and migration of muscle precursor cells.

The proliferation of myoblast is regulated by Mrfs: Myf5, MyoD and Mrf4. Myogenin and MyoD control the differentiation of myoblast and their fusion into myotubes. Mrf4 plays an important role in termination of
differentiation and formation of muscle fibres. All these factors are also regulated: Mox2 regulates Pax3 and Myf5 and the Six homeo domains (Six 1 and Six 4) regulate the transcription of MyoD, while Pax3/7 regulate MyoD and Myf5. Pax7 is also essential for the formation and survival of satellite cells. The transcription factor, Msx1, and members of fibroblast growth factor family, such as FGF8 and FGFR4, are involved in migration of precursor cells and proliferation of myoblasts. There are also several signalling molecules, including Sonic hedgehog (Shh), Wnt proteins, and bone morphogenic proteins (BMPs), involved in muscle development. The Mrfs form heterodimers by interacting with members of the E family and the myocyte enhancer factor 2 (MEF2) to activate the transcription of muscle specific genes such as α-actin, myosin heavy chain (MyHC) or muscle creatine kinase (MCK). p38 mitogen activated protein kinase (p38 MAPK) is essential for myoblast differentiation and also controls the transcription of the muscle specific genes.

Structure

Macroanatomy

Skeletal muscles have different sizes, shapes, and arrangement of fibres to accomplish different functions. The muscle belly can be split into two main components: the muscle fibres (myofibres) that are responsible for the contractile activity, and the connective tissue (composed of collagen and elastin fibres) that keeps the structure of the muscle belly and transmits the contractile force produced in the fibre to the tendon and, consequently, to the bone. There are several layers of muscle connective tissue: 1) the fascia that covers the entire muscle, 2) the epimysium, a thin layer surrounded the whole muscle, 3) the perimysium that wraps a bundle of muscle fibres (or fascicle) and where the neuromuscular bundle (arteries, veins and nerve) run longitudinally, and 4) the endomysium that separates individual muscle fibres (figure 1).

Each muscle fibre (10-100 µm diameter) is a single cell formed by the longitudinal fusion of numerous myoblasts and, therefore, has many nuclei. Around each cell is the plasma membrane called the sarcolemma and the basement membrane (extracellular matrix) composed of the basal lamina and the reticular lamina. Located between the sarcolemma and the basal lamina are quiescent satellite cells, which undertake postnatal skeletal muscle growth and regeneration, waiting to be activated in case of muscle injury (figure 1).
Individual muscle fibre is made up of myofibrils (cylindrical units that run the entire length of the fibre), the sarcoplasmic reticulum, mitochondria, and transverse tubular systems. Myofibrils comprise about 90% of the fibre (in a fibre of 50 μm diameter, there are up to 8,000 myofibrils). The sarcoplasmic reticulum is responsible for the Ca^{2+} secretion and uptake in the fibre. Mitochondria control the production of ATP, the energy used during contraction. 

**Microanatomy**

Each myofibril (1-2 μm diameter) is made up of repeated contractile units called sarcomeres. One mammalian sarcomere is ~2 μm in length. The sarcomeres are formed of the transverse I- and A- bands and the longitudinal Z-disk and M-line. Actin (thin filament) is the main component of the I-band (the light band) that also contains the Z-disk. Myosin (thick filament) is the main component of the A-band (the dark band) that also comprises the M-line and the area where myosin and actin interact. Major multicompartment proteins in the sarcomere are summarized in figure 2, although new proteins are continually being identified. It has recently been found that several sarcomeric proteins, such as the muscle-specific RING finger protein-1/-2 (MuRF-1/-2), myopodin, and ankyrin repeat domain 2 (Ankrd2), translocate to the nucleus in response to stress, i.e., stretch, heat-shock or atrophic conditions.
Figure 2. Schematic representation of the sarcomere. Actin filaments are regulated by the tropomyosin–troponin complex, and thick filaments by the regulatory myosin-binding proteins-C and -H. The sarcomere also contains numerous proteins with multiple localizations and with the potential to exchange between the Z-disks, I-bands and M-bands (green arrows), as well as to translocate to the nucleus (pink arrows). With permission from Elsevier.

During myofibrillogenesis newly synthesized proteins are subjected to quality control in order to ensure proper muscle formation. This process is regulated by the ubiquitin-proteasome system (UPS) and molecular chaperones. The chaperones TCP-1 Ring Complex (TRiC) and prefoldin/GimC regulate actin folding and assembly, whereas UNC45 and heat shock proteins 90 and 70 (HSP90, HSP70) have the same function concerning myosin.

Contractile proteins

Actin

Actin is a globular (G-actin, ~42 kDa) and ubiquitous protein composed of 375 amino acids and 4 subdomains. It is the most abundant protein in the thin filament and has been highly conserved among species throughout evolution. There are two sarcomeric, striated muscle actins: α-skeletal
muscle and α-cardiac muscle, which are co-expressed to varying degrees in skeletal and cardiac muscles. Actin filaments are formed of two strands of actin polymers arranged in a double helix structure termed F-actin. The regulatory proteins, tropomyosin and troponins, are attached to the actin filament. Furthermore, actin has a 2-fold protein turnover rate longer than myosin.

It has been demonstrated that muscle unloading and/or the absence of weight bearing, i.e., after bed rest, exposure to microgravity or hind-limb suspension, are the major factors decreasing α-actin at transcriptional and protein levels.

Myosin
Muscle myosin belongs to the myosin family II. The thick filaments are composed of ~600 molecules of myosin. Each myosin filament is surrounded by 6 actin filaments. Myosin (~500 kDa) is the motor protein of the sarcomere and generates muscle contraction by converting chemical energy into mechanical energy via ATPase activity in order to move along actin filaments. Myosin is comprised of two myosin heavy chains (MyHC, ~220kDa) and four myosin light chains (MyLC, ~20kDa), which are divided into two essential light chains (ELC) and two regulatory light chains (RLC). The myosin molecule can be divided into two domains: the head and the rod. The head domain (the S1 fragment) is composed of the N-terminal region of each MyHC and two MyLCs (one essential and one regulatory) and contains the binding sites for actin and for ATP molecule that will be hydrolysed. The C-terminal regions of the two MyHC form the α-helical coiled-coil rod. The part of the rod that connects S1 to the coiled-coil rod is referred to as the S2 fragment. After proteolytic cleavage, myosin is split up into heavy meromyosin (HMM) and light meromyosin (LMM). Further cleavage of HMM separates the single-headed S1 fragment from the S2 fragment.

There are different kinds of muscles with different requirements; therefore, there are several MyHC and MyLC isoforms. At least, there are nine muscle MyHC isoforms in mammals, each encoded by specific genes: MyHC-I (or MyHCβ/slow) is expressed in the ventricle of the heart and in slow skeletal muscle fibres; MyHC-IIa, MyHC-IIx/IId, and MyHC-IIb are expressed in fast skeletal muscle fibres; MyHC-α, a slow isoform, expressed in the atria of heart muscle, and in extraocular and masticatory muscles; MyHC-exoc and MyHC-IIm in extraocular and masticatory muscle fibres, respectively; and MyHC-emb and MyHC-neo, expressed in muscles at different developmental stages. The main characteristics for the MyHC-I and MyHC-II fibres are listed in Table 1.
Table 1. Main characteristics of adult skeletal muscle myosin heavy chain (MyHC)

<table>
<thead>
<tr>
<th>MyHC isoform type</th>
<th>MyHC-I</th>
<th>MyHC-IIa</th>
<th>MyHC-IIx/IId</th>
<th>MyHC-IIb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contraction velocity</td>
<td>Slow</td>
<td>Moderately fast</td>
<td>Fast</td>
<td>Very fast</td>
</tr>
<tr>
<td>Size of motor neuron</td>
<td>Small</td>
<td>Medium</td>
<td>Large</td>
<td>Very large</td>
</tr>
<tr>
<td>Myoglobin content</td>
<td>High (red)</td>
<td>Fairly high</td>
<td>Intermediate</td>
<td>Low (white)</td>
</tr>
<tr>
<td>Resistance to fatigue</td>
<td>High</td>
<td>Fairly high</td>
<td>Intermediate</td>
<td>Low</td>
</tr>
<tr>
<td>Activity Used for</td>
<td>Aerobic</td>
<td>Long-term anaerobic</td>
<td>Short-term anaerobic</td>
<td>Short-term anaerobic</td>
</tr>
<tr>
<td>Endurance</td>
<td>High</td>
<td>Fairly high</td>
<td>Intermediate</td>
<td>Low</td>
</tr>
<tr>
<td>Power produced</td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
<td>Very high</td>
</tr>
<tr>
<td>Mitochondrial density</td>
<td>Very High</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Myonuclear density</td>
<td>Very High</td>
<td>High</td>
<td>Medium</td>
<td>Low</td>
</tr>
<tr>
<td>Cross-sectional area</td>
<td>Small</td>
<td>Medium</td>
<td>Large</td>
<td>Very large</td>
</tr>
<tr>
<td>Capillary density</td>
<td>High</td>
<td>Intermediate</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Oxidative capacity</td>
<td>High</td>
<td>High</td>
<td>Intermediate</td>
<td>Low</td>
</tr>
<tr>
<td>Glycolytic capacity</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>High</td>
</tr>
</tbody>
</table>

Different factors influence the MyHC isoform composition of a muscle fibre such as innervation/neuromuscular activity, exercise training, mechanical loading/unloading, hormones and aging. Generally, fibre type transitions follow a serial pattern from fast to slow and slow to fast: MyHC-I ↔ MyHC-IIa ↔ MyHC-IIx/IId ↔ MyHC-IIb\textsuperscript{25,26}.

As it has been demonstrated, myosin is constantly turning over and the half-life for MyHC is between 7 to 10 days in normal conditions\textsuperscript{27}.

Myosin binding proteins

MyBP-C (~140 kDa) and MyBP-H (~58 kDa) are myosin binding proteins and both are components of the thick filament located in the C-zone of the A-band, forming transverse stripes. They are made up of a series of globular motifs (repetitions of fibronectin and Ig domains), each composed of 90-100 amino acids. In adults there are three isoforms of MyBP-C encoded by different genes: skeletal fast, skeletal slow and cardiac, and one embryonic isoform. MyBP-C has two myosin- and one titin-binding site. In contrast, there is only one isoform of MyBP-H expressed in the Purkinje fibres of the heart and in fast skeletal muscle fibres. MyBP-H has one myosin-binding site\textsuperscript{11}. Skeletal MyBP-C has 10 globular domains whereas MyBP-H is composed of a unique N-terminal sequence of 131 amino acids followed by 4 globular domains, which arrangement is identical to the latest four C-terminal domains of MyBP-C. It has been demonstrated that MyBPs interact with the thick filament through these common four C-terminal domains\textsuperscript{28}.
To date, the precise functions of MyBPs are still uncertain, but MyBP-C appears to play an important role in the assembly of thick filaments during myofibrillogenesis and regulation of muscle contraction\textsuperscript{29,30}. The role of MyBP-H in the muscle is unclear. The C-terminus of MyBP-H is essential for the formation of long myosin “cables”\textsuperscript{29}. Mutations in the cardiac isoform of MyBP-C are one of the major causes of hypertrophic cardiomyopathy; since many of these patients have a mild phenotype, it has been suggested that up-regulation of MyBP-H may compensate MyBP-C insufficiency\textsuperscript{28}. A recent study using non-muscle carcinogenic cells showed that MyBP-H negatively regulates actomyosin organization, reducing cell motility, invasion, and metastasis through ROCK1 inhibition\textsuperscript{31}.

**Muscle contraction**

Muscle contraction is regulated by the central nervous system. Muscles are activated by lower motor neurons that end at the neuromuscular junction. When a nerve impulse arrives at the neuromuscular junction, the neurotransmitter, acetylcholine (ACh) is released. Binding of ACh to the postsynaptic nicotinic ACh receptors opens specific ion channels in the sarcolemma allowing sodium ions to enter the cytosol and potassium ions to leave the cell. Since more sodium ions enter, the muscle fibre membrane becomes more positively charged, triggering the depolarisation of the membrane and, consequently, the action potential. The action potential travels along the length of the fibre and activates the contractile machinery. To reach all sarcomeres in the fibre, the sarcolemma has invaginations called T-tubuli that run at the border of each sarcomere between the A- and the I-band. On the wall of the T-tubuli there are depolarisation sensitive Ca\textsuperscript{2+} channels (dihydropyridine receptors, DHPR) that are mechanically coupled to other Ca\textsuperscript{2+} channels (ryanodine receptors, RyR) in the adjacent sarcoplasmic reticulum (SR). The action potential activates DHPRs, which in turn activate the RyRs to realise Ca\textsuperscript{2+} from the SR. The membrane of the SR has calcium-dependent ATP pumps that release Ca\textsuperscript{2+} from/to sarcoplasm. In the relaxed state, the myosin-binding site on actin is covered by tropomyosin, then when the SR releases Ca\textsuperscript{2+} into the cytoplasm it binds to troponin C, which produces conformational changes on the binding sites of actin, displacing tropomyosin and allowing the interaction between actin and myosin\textsuperscript{32}. During contraction, myosin filaments slide longitudinally past the actin filaments, which move toward the centre of the sarcomere, causing the I-band shortening (sliding filament theory)\textsuperscript{33}.

The acto-myosin interaction is initiated by a weak interaction between positively charged residues on the surface of the myosin head and negatively charged residues on the surface of actin. When ATP binds to the globular head of MyHC, myosin detaches from the actin allowing the globular head...
to hydrolyse ATP to ADP and Pi. The liberated energy is used to generate force: Pi is released, the globular head is turned around its axis and attaches to an actin molecule seven monomers upstream (the power stroke)\textsuperscript{34}. This cyclic process is known as the cross-bridge theory: myosin filament interacts cyclically in a rowing motion with the actin filament when ATP is hydrolysed.

The number of cross-bridges and the force generated per cross bridge determine the amount of force generated by the whole muscle fibre\textsuperscript{35,36}. The duration of a cross-bridge cycle depends on temperature, MyHC isoform, and speed of contraction and can vary from less than 1 ms to 50 ms. The duration of the isometric twitch is related to the amount and speed of Ca\textsuperscript{2+} released, which is more efficient in fast than in slow fibres\textsuperscript{37}.

### Intensive care unit muscle wasting

The first reported case about muscular weakness in critical illness was described by Osler\textsuperscript{38} in 1892 as a “rapid loss of flesh” in patients with prolonged sepsis. There is a wide range of neuromuscular disorders-muscle wasting conditions seen in the intensive care unit (ICU). However, it is necessary to distinguish between those diseases that cause sufficient weakness to produce ICU admission, such as severe myasthenia gravis or the Guillain-Barré syndrome, and those diseases that are a consequence of the ICU intervention and are consequently developed during ICU stay below the primary disease such as critical illness polyneuropathy (CIP) or acute quadriplegic myopathy (AQM).

The reported prevalence of ICU-acquired weakness depends on the method of assessment, patient population and timing of evaluation, but it has been reported to vary between 25% and 58% in ICU patients mechanically ventilated for more than one week\textsuperscript{39,40}. In certain ICU sub-groups, i.e., ICU patients with sepsis, the prevalence is higher: between 50% and 100\%\textsuperscript{41-43}. The muscle wasting and weakness developed by ICU patients has devastating consequences: prolonged ICU stay and failure to wean from ventilator, increasing financial cost, morbidity and mortality and a decreasing quality of life (QoL) of survivors. Moreover, the impairment in muscle function may persist 5 years after hospital discharge\textsuperscript{44-46}. One of the most frequent neuromuscular disorders underlying the muscle weakness and/or paralysis in ICU patients is AQM.
Acute quadriplegic myopathy

In 1977, MacFarlane and Rosenthal\textsuperscript{47} reported a case of acute quadriplegia affecting spinal nerve innervated muscles and with intact sensory, cognitive and craniofacial muscle function in a 24-year-old woman after the treatment of a status asthmaticus attack with high doses of intravenous corticosteroids (CS), mechanical ventilation and non-depolarizing neuromuscular blocking agents (NMBAs). The weakness remained two months after ICU discharge. Several different names have been given to this myopathy such as “thick filament myopathy”, “critical illness myopathy”, “prolonged reversible quadripareisis”, “acute myopathy in severe asthma” and “myopathy of intensive care”\textsuperscript{48}. However, the term to deserve this condition is acute quadriplegic myopathy (AQM) since it is the primary name and reflects the hypo/areflexic quadriplegia generally seen in these patients\textsuperscript{49}.

AQM is a common and acquired neuromuscular disorder seen in ICU patients and is characterized by acute muscle wasting and persistent symmetric weakness, especially affecting limb muscles, and consequently impaired muscle function\textsuperscript{48}. AQM was for many years considered to be rare and of limited clinical significance due to miss- or under-diagnosis. The prevalence rates of AQM are not clear and vary in the literature, although some studies indicate that it can be developed by 36-42\% of the ICU population\textsuperscript{50,51}.

Although the exact causes of AQM are still unknown, immobilization, prolonged mechanical ventilation, use of NMBA and/or CS have been forwarded as important triggering factors. Sepsis, organ transplantation, multi-organ failure, and hyperglycemia are also speculated risk factors for this myopathy\textsuperscript{48,52,53}.

The effects of AQM go further than just muscle weakness, it also can cause increased ICU stay, higher morbidity rates, and impaired rehabilitation. Furthermore, the median hospital charge (excluding the prolonged rehabilitation process) for AQM patients is $91,476, i.e., in excess of $66,713 as compared to ICU control patients\textsuperscript{54}.

Features

AQM is predictable in patients with weaning difficulties 7–10 days after mechanical ventilation and ICU treatment\textsuperscript{55}. The major characteristics are diffuse flaccid weakness (especially affecting limb muscles), a marked atrophy of skeletal muscle (defined as a decrease in muscle cross-sectional area (CSA) or mass), preserved or decreased deep tendon reflexes, and failure to wean from the respirator. Electrophysiological analyses has demonstrated a decreased compound muscle action potential (CMAP), short
duration and low motor unit potential (MUP), and an intact sensory nerve action potential (SNAP)\textsuperscript{56-58}. More specifically the muscle membrane in AQM presents decreased excitability, or in-excitability, due to an increase of inactivated sodium channels\textsuperscript{59,60}. A preferential loss of myosin and myosin associated proteins is considered a hallmark of AQM\textsuperscript{61,62}. Signs of AQM can be detected as early as 4 days in ICU\textsuperscript{63}. Clinical, electrophysiological, and histological features of AQM\textsuperscript{56,57,64} are summarized in Table 2.

### Table 2. Clinical, electrophysiological, and histological features of AQM

<table>
<thead>
<tr>
<th>Features of AQM</th>
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<tr>
<td><strong>Physical examination</strong></td>
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<tr>
<td>Critically ill (multiorgan dysfunction and failures)</td>
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<td>Persistent symmetric weakness especially affecting limb muscles</td>
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<tr>
<td>Muscle atrophy</td>
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<td>Difficulty of weaning from ventilator</td>
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<tr>
<td>Preserved or decreased deep tendon reflexes</td>
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<tr>
<td>No sensory deficits</td>
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<tr>
<td>Cranial nerves usually spared</td>
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<tr>
<td>Rarely facial and extra-ocular weakness</td>
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<tr>
<td><strong>EMG</strong></td>
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<tr>
<td>Reduced muscle membrane excitability</td>
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<tr>
<td>Decreased CMAP amplitude and increased duration</td>
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<tr>
<td>Short duration and low MUP amplitude with early or normal recruitment, with or without fibrillation potentials</td>
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<tr>
<td>Intact SNAP</td>
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<tr>
<td><strong>Muscle biopsy</strong></td>
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<tr>
<td>Preferential myosin loss</td>
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<tr>
<td>Necrosis and regeneration of muscle fibres may be present</td>
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<tr>
<td>Inflammatory infiltrate and vacuoles may be present</td>
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<tr>
<td><strong>Laboratory test</strong></td>
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<tr>
<td>Normal or elevated serum CK</td>
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EMG, electromyography; CMAP, compound muscle action potential; MUP, motor unit potential; SNAP, sensory nerve action potential; CK, creatine kinase

### Diagnosis

The diagnosis of AQM is not trivial and requires specialized neurophysiological methods, such as direct muscle stimulation (DMS) and/or biopsies, in addition to nerve conduction studies and needle electromyography. There have been proposed diagnostic criteria for AQM\textsuperscript{48,64,65} which include: 1) SNAP amplitudes >80% of the lower limit of
normal in two or more nerves, 2) CMAP amplitudes <80% of the lower limit of normal in two or more nerves without conduction block, 3) needle EMG with short-duration, low amplitude MUPs with early or normal full recruitment with or without fibrillation potentials in two or more muscle groups; or increased CMAP duration, 4) reduced muscle membrane excitability on DMS, 5) absence of a decremental response on repetitive nerve stimulation, and 6) muscle histopathologic findings of myopathy with myosin loss. An elevation of serum creatine kinase (CK) has been reported in some patients during the first week (later CK returns to normal values) and can be considered as a supportive diagnosis feature.

In spite of these criteria, a correct diagnosis of AQM is still difficult for several reasons: 1) major EMG criteria requires the cooperation of the patient and most ICU patients are unable to voluntary contract muscle due to severe weakness, deep sedation, neuromuscular blockade or encephalopathy. In these cases, EMG cannot differentiate between myopathy and neuropathy, 2) the co-existence of AQM and CIP makes it more difficult to make the appropriate diagnosis, and 3) there are several independent factors that complicate the diagnosis of AQM such as the origin of primary disease, different pharmacological treatments, muscle biopsies taken several weeks after ICU admission, and exposure to different causative agents.

CIP is a diffuse, symmetric, and distal axonal sensory-motor polyneuropathy affecting limb and respiratory muscles, and it is the most frequent polyneuropathy in ICU. EMG poorly distinguishes between both disorders in non-cooperative patients. Hence in many previous years and still now, AQM and CIP have been overlapped, resulting in incorrect diagnosis and interfering in the prognosis of both disorders. Moreover, AQM and CIM can co-exist. DMS can help to differentiate between CIM and CIP: nerveCMAP/muscleCMAP ratio <0.5 indicates neuropathy, and >0.5 in combination with reduced muscleCMAP amplitudes indicate myopathy and an impaired muscle membrane excitability is suggested. An original algorithm has recently been proposed for better distinguishing between AQM and CIP, taking into account first the amplitude of the responses to DMS, then the nerveCMAP/muscleCMAP ratio, and finally the amplitude of the SNAPs. However, DMS requires specialized and experienced clinical staff to achieve and interpret results in a reliable way.

A definitive diagnosis of AQM requires histologic confirmation with a muscle biopsy, although it is an invasive procedure. Muscle biopsy reveals a selective loss of myosin and myosin associated proteins (a factor pathognomonic of AQM), muscle fibre atrophy with preferential loss of type II fibres, and occasional fibre necrosis and regeneration. Widespread
myosin loss can be detected by different methods such as electron microscopic, enzyme- and immune-cytochemical analyses of muscle biopsy cross-sections, and gel electrophoresis. Among all these techniques, electrophoretic separation of myofibrillar proteins by horizontal SDS-PAGE and measurement of myosin:actin ratio has been suggested as a good, sensitive and rapid diagnostic tool for AQM, detecting decreased myosin:actin ratio in patients with AQM71.

Risk factors

Immobilization

It has been demonstrated that in critically ill patients the muscle atrophy starts within 4 hours of bed rest and produces changes in muscle morphology, although the rate of muscle atrophy will depend on the muscle conditions prior to hospitalization72. In healthy individuals, each week of bed rest produces a 4-5% loss of muscle strength and 3% loss of muscle mass73. Moreover, immobilization induces a shift from slow fibres (type I) to fast fibres (type II) that are less fatigue resistant25. In addition, immobilization increases pro-inflammatory cytokines74 and the production of reactive oxygen species (ROS)72, resulting in more muscle proteolysis, therefore, impaired muscle function and atrophy75,76.

Corticosteroids

The use of CS in the ICU is widely debated, balancing benefits during sepsis, airway oedema, and autoimmune disorders against negative effects such as increased rates of infection and prolonged mechanical ventilation and ICU stay77. There is conflicting data regarding the association between CS and AQM: a prospective study pointed out corticosteroids as a key factor in developing muscle weakness in ICU39, however, other studies showed no consistent relationship between corticosteroids and the development of AQM/CIP78,79.

Neuromuscular blocking agents

NMBAs are used with sedatives and/or analgesics to facilitate mechanical ventilation in some critically ill patients and improve arterial partial pressure of oxygen. There is a trend towards reducing the use of NMBAs in ICU since they are associated with a longer ICU stay, longer duration of mechanical ventilation and higher mortality and morbidity80, although a recent study showed that this association is not clear and use of NMBAs may be even beneficial, decreasing time on the ventilator, mortality and morbidity81. Approximately 13% of ICU patients receive NMBAs for at least one day82 and in some ICU sub-groups, e.g., patients with acute respiratory distress syndrome (ARDS), the incidence is typically higher. The effect of NMBAs on AQM shows contradictory results: there have been reports of the
prolonged weakness and/or paralysis after long term and/or high doses NMBA$^{79,83}$ and it seems that the incidence of prolonged weakness increases when NMBAs are combined with CS$^{61,84,85}$. However, other reports demonstrated that AQM is no related to NMBA$^{39,86}$ and/or CS$^{87,88}$.

**Sepsis**

Systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis and septic shock all define gradual stages of disease severity that correlate with mortality. SIRS is an unspecific inflammatory host response due to both infectious and non-infectious origins. Severe sepsis refers to a systemic infection associated with acute organ dysfunction and septic shock occurs if in addition, there is volume-refractory hemodynamic failure$^{89}$. Mitochondrial dysfunction has been correlated to septic shock$^{90}$. SIRS and sepsis have both been indicated as likely crucial risk factors for development of AQM$^{86,91-93}$. The pathophysiology of sepsis in AQM may be regarded as a channelopathy affecting the inactivation of Na$^+$ channels$^{93}$.

**Other risk factors**

There are a number of speculated factors related to AQM such as female sex, older age, acute asthma, exacerbation of chronic obstructive pulmonary disease, prolonged ICU stay, duration of mechanical ventilation, multi-organ failure, organ transplantation, and hyperglycemia$^{52,86,94,95}$.

**Treatment**

To date, there is no specific and effective treatment for AQM, only preventive and supportive strategies. It seems prudent to avoid or use the minimal doses for as short period as possible of CS and/or NMBAs and try to avoid/treat sepsis in ICU patients. Novel strategies for the treatment of sepsis have been developed$^{96}$.

In an attempt to reduce the incidence and severity of AQM, nutritional and supplemental strategies have been targeted, including amino acid supplementation, antioxidant therapy and hormonal therapy$^{97}$. Administration of the antioxidant glutathione (GSH) together with acetilcysteine decreases oxidative stress levels in ICU patients with septic shock$^{98}$. Glutamine supplementation, in especially high doses and administered via parenteral, may decrease infectious complication, ICU stay and mortality rates$^{99}$. However, so far there has been no attempt to examine these components in treating/preventing AQM yet. Exogenous administration of Growth Hormone (GH) decreases muscle protein catabolism and urea generation in ICU patients but with regards to muscle function, results are inconsistent$^{100-102}$; moreover, GH administration could increase mortality during the acute phase of critical illness$^{103}$. Some studies suggest that intensive insulin therapy reduces the incidence of AQM/CIP and
ventilator days\textsuperscript{104-106}; however, questions concerning to safety, risk of hypoglycemia, and further limitations of the studies due to different diagnostic criteria used are still debated and thus these beneficial effects may be overestimated\textsuperscript{107}.

Early mobilization has been suggested as the best strategy to prevent muscle atrophy, reducing ventilator days and the length of stay in ICU, as well as enhancing muscle force, functional outcomes and recovery in ICU patients\textsuperscript{108-111}. Since active movement is generally not possible in these patients due to immobilization and deep sedation, passive movement has arisen as an alternative to slow the progression of muscle atrophy and improve the functional outcomes. A clinical study with five ICU patients mechanically ventilated and treated with NMBAs, demonstrated that passive stretching for three hours, three times per day over a period of seven days, can preserve muscle fibre size, protein loss, and muscle architecture with less cell necrosis\textsuperscript{112}. Other studies suggested that daily electrical muscle stimulation (EMS) sessions can prevent the development of AQM/CIP in critically ill patients and maintain their muscle mass and strength\textsuperscript{113,114}.

Regulation of skeletal muscle mass

Skeletal muscles have an extreme plasticity in order to adapt themselves to different circumstances and accomplish the wide functional demands. They can vary their properties by changing the protein expression, either by the amount of protein expressed and/or the type of protein isoform. Changes in muscle mass and type alter muscle function, including the amount of force generated during contraction, the speed of shortening, and the resistance to fatigue.

The maintenance of skeletal muscle mass is determined by a fine balance between protein synthesis and protein degradation. Muscle hypertrophy is the process of muscle gain caused by increased protein synthesis and a simultaneous decrease in protein degradation, and can be acquired in response to growth factors and exercise\textsuperscript{115}. The loss of muscle mass (muscle atrophy) is mainly due to a loss of contractile proteins, and can occur in a large number of conditions including muscle disuse, microgravity conditions, cast immobilization, denervation, chronic kidney disease, cachexia, aging, sepsis, diabetes, and spinal cord injury\textsuperscript{115-117}. Synthesis and degradation are regulated by several signalling pathways. Once the stimulus occurs, signalling pathways are activated and the cascade of events that results in protein synthesis, is initiated. Protein synthesis is regulated at several levels: transcriptional, post-transcriptional, pre-translational,
translational and post-translational (figure 3). The product of each step is subjected to degradation control.

![Diagram of genetic information flow](image)

**Figure 3. Flow of genetic information leading to protein synthesis.**

**Signalling pathways**

**Akt signalling pathway**

The Akt signalling pathway is one of the most important pathways regulating atrophy and hypertrophy. Akt activation is triggered by insulin growth factor-1 (IGF-1) and insulin, which activate insulin receptor substrate (IRS). Activated IRS in turn recruits and activates phosphatidylinositol 3-kinase (PI3K) to generate phosphatidylinositol triphosphate (PIP3). PIP3 translocates Akt to the plasma membrane by binding to its NH2-terminal pleckstrin homology domain. Akt is then phosphorylated at one threonine site by phospho-inositide dependent kinase 1 (PDK1) and one serine site by one of the mTOR complexes (mTORC2). Akt has three isoforms in mammals encoded by different genes: Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ). Akt1 and Akt2 are expressed in almost all tissues and organs at higher levels than Akt3, which is mainly expressed in brain and testis. Akt2 is the dominant isoform in skeletal muscle, fat and liver (insulin responsive tissues) at the gene level. Studies using Akt mutant mice demonstrated that both Akt1 and Akt2 are important for animal growth; moreover, Akt1/2 double mutant mice are smaller than single knockout mice and present impaired skin and bone development, and skeletal muscle atrophy. Furthermore, Akt activation for three weeks is enough to produce a marked
hypertrophy, independently of satellite cell activation\textsuperscript{121}. It seems that insulin activates Akt2 whereas IGF-1 and exercise \textit{in vivo} primarily activate Akt1\textsuperscript{116}. Muscle contractile activity, i.e., exercise or mechanical loading, can increase Akt activity. However, this effect could be fibre type dependent since it was observed only in the fast-twitch muscle extensor digitorum longus (EDL), but not in the slow-twitch soleus from rats in response to stretch\textsuperscript{116,122}. Contrariwise, under atrophic conditions, such as disuse, burn or hind-limb suspension, Akt activity is decreased\textsuperscript{115,123}. Interestingly, in hibernating animals, which maintain their muscle mass despite of long periods of immobilization, Akt activity is also suppressed, suggesting that there are other mechanisms involved in preventing muscle mass loss in these animals\textsuperscript{124}.

Akt has two downstream effectors that stimulate protein synthesis and hence hypertrophy: mammalian target of rapamycin (mTOR) and the glycogen synthase kinase 3 beta (GSK3-\beta), and one downstream target that mediates protein degradation and hence atrophy: forkhead box O (FoxO)\textsuperscript{123}. An overview of the Akt signalling pathway is shown in figure 4.

\textit{mTOR}

mTOR is part of two multiprotein complexes: mTORC1, associated with raptor, rapamicin sensitive, and is responsible for downstream signalling, negatively regulating Akt pathway via S6K1. mTORC2 is associated with rictor, and is responsible for Akt phosphorylation, activating Akt signalling\textsuperscript{116}. Amino acids can also activate mTOR; consequently, deprivation of amino acids (especially leucine and isoleucine) can inhibit mTOR, which is also suppressed by rapamycin\textsuperscript{115}. Moreover, AMP, a key component for regulating energy homeostasis in the cell, also affects mTOR via AMPK\textsuperscript{125}. To all of these, mTOR has been arisen as an important factor in integrating growth signals, and nutritional/energy status to enhance protein synthesis and cell growth\textsuperscript{116,125}.

The activation of mTORC1 by Akt leads to phosphorylation of the ribosomal protein S6 kinase (S6K1), which in turn phosphorylates the ribosomal protein S6, inducing protein anabolism. Furthermore, S6K1 is necessary for myofibre growth since mice S6K1(-/-) show smaller myotubes with normal myonuclear number\textsuperscript{126}, and S6K1 also inhibits AMPK and PI3K activation\textsuperscript{116,127}. mTORC1 also phosphorylates the eukaryotic initiation factor 4E binding protein 1 (4eBP1, also known as PHAS-1), leading to its inhibition. 4eBP1 is a negative regulator of eukaryotic initiation factor 4E (eIF4e), a translation initiation factor; consequently, when 4eBP1 is inhibited, eIF4e is released, stimulating protein synthesis\textsuperscript{115}. The Akt/mTOR pathway is sufficient to cause hypertrophy and can prevent atrophy \textit{in vivo}.
Glucocorticosteroids, which are elevated in muscle atrophy conditions, are able to decrease IGF-1 expression and inhibit mTOR via REDD1\textsuperscript{116}.

**GSK3-β**

Phosphorylation of GSK3-β by Akt results in its inhibition, leading to increased activity of eIF2b, another translation initiation factor that stimulates protein synthesis. Expression of the dominant inactive form of GSK3-β or its inhibition, induces hypertrophy in skeletal myotubes, independent of mTOR\textsuperscript{115}. GSK3-β is also inhibited through Wnt signalling, promoting hypertrophy, cell proliferation and myoblast differentiation. This effect was reinforced when cells were co-treated with insulin, suggesting that insulin and Wnt pathways cooperate to induce myotube hypertrophy and have a common target, GSK3-β\textsuperscript{129}. In addition, GSK3-β inhibition contributes to actin filament formation and assembly via nebulin/neuronal Wiscott-Aldrich syndrome protein (N-WASP)\textsuperscript{130}.

**FoxO**

In skeletal muscle there are three FoxO isorforms: FoxO1, FoxO3, and FoxO4. Active Akt suppresses protein degradation by inactivating FoxO: Akt phosphorylates FoxO, which is translocated from the nucleus to the cytoplasm, resulting in decreased transcription of the atrogenes (genes associated with atrophy) including MuRF1 and atrogin-1\textsuperscript{115,128}. FoxO1 or 3 are sufficient to cause muscle atrophy \textit{in vivo} and \textit{in vitro}\textsuperscript{116}. Moreover, FoxO also activates 4eBP1 and down-regulates both RAPTOR and mTOR. AMPK and ROS can also activate FoxO, independently of the Akt pathway\textsuperscript{116,123}. Overexpression of peroxisome proliferator activated receptor γ coactivator 1α (PGC1α), that regulates mitochondria homeostasis, blocks the muscle atrophy induced by FoxO/atrogin-1\textsuperscript{116}.

**Proteolytic pathways**

*The ubiquitin-proteasome system (UPS)*

The UPS recognizes specific protein substrates and marks them for subsequent degradation by the proteasome, following the coordinated activation of three enzymes: ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2) and ubiquitin ligase (E3). UPS is the major contributor to muscle proteolysis\textsuperscript{131} and, in addition, it is also involved in protein quality control, degrading non-functional, misfolded or damaged proteins\textsuperscript{132}. Atrogin-1 (F-box protein 32 or MAFbx) and MuRF1 (tripartite motif-containing 63 (Trim63)) are two muscle-specific E3s that are responsible for the ubiquitination and subsequent degradation of muscle proteins under atrophic conditions\textsuperscript{115}. MuRF1 degrades thick filaments while atrogin-1 has MyoD, calcineurin and the translation initiation factor eIF3f as
substrates\textsuperscript{115,133,134}. Several signalling pathways have been associated with the activation/inactivation of MuRF1 and atrogin-1.

The autophagy-lysosome system (ALS)
Cathepsins are lysosomal proteases that are capable of degrading a variety of extra- and intra-cellular proteins and organelles such as mitochondria\textsuperscript{135}. ALS has been implicated in muscle wasting conditions including muscular dystrophies, sepsis, disuse atrophy, fasting, and glucocorticoid induced atrophy. Moreover, AMPK activates autophagy whereas mTOR inhibits it\textsuperscript{136}. FoxO3 is required to activate ALS, stimulating protein degradation\textsuperscript{137}. Excessive autophagy is detrimental to muscle mass and impaired autophagy leads do different myopathies. However, an optimum ALS is essential for muscle tissue homeostasis since it removes dysfunctional mitochondria and also mediates the positive effects of exercise on metabolism\textsuperscript{138,139}.

The calpain system
Calpains are calcium activated proteases that are capable of cleaving but not degrading sarcomeric proteins such as actin, myosin, titin, nebulin, desmin, and α-actinin, which after being released, are degraded by UPS\textsuperscript{140}. Muscle tissue expresses mainly three different types of calpains: the ubiquitous calpains-1, -2 (mu and m) and muscle specific calpain-3\textsuperscript{141}. Oxidation can activate calpain-1 and -2, promoting degradation of myofibrillar proteins\textsuperscript{142}. Calpain-3 has been found down-regulated in several atrophic conditions and is thus not likely to participate in protein degradation. Therefore, it has been indicated that calpain-3 may have a protective function against atrophy by addressing the NF-κB pathway towards an anti-apoptotic response or through adjustment of proteasome activity. Moreover, the deficit of calpain-3 leads to a muscular dystrophy, suggesting a role of calpain-3 in muscle regulation and homeostasis\textsuperscript{141}.

The caspase system
Apoptosis is orchestrated by a group of proteases named caspases (cysteiny1 aspartate proteases) that are activated under long periods of muscle wasting. The activation of caspases is controlled by pro- and anti-apoptotic family members, such as Bcl-2 family (anti-apoptotic), Bax and Bod (pro-apoptotic), heat shock proteins, and inhibitors of apoptosis proteins\textsuperscript{143}. Caspases can be divided into initiator caspases: caspases -2, -8, -9 and -10 and executioner caspases: caspases -3, -6 and -7. Initiator caspases activate executioner caspases that cleave a large number of substrates in the cell such as cytoskeletal components, cell adhesion molecules, DNases that degrade DNA, translational proteins and lamins on the nuclear envelope\textsuperscript{144}. Caspase-3 degrades actinomyosin, leaving 14 KDa actin fragment\textsuperscript{143}.
NF-κB pathway

This pathway is activated in skeletal muscle during disuse, sepsis and cachexia. NF-κB is also a negative regulator of myogenesis. NF-κB activation is triggered by inflammatory cytokines such as tumour necrosis factor alpha (TNFα) and TNF-related weak induced of apoptosis (TWEAK). In mammals there are five NF-κB transcription factors, all of them expressed in skeletal muscle: p65 (RelA), RelB, c-rel, p50/p105 (NF-κB1), and p52/p100 (NF-κB2). They usually form heterodimers that bind to IκB (inhibitor of κB) proteins. Upon cachexia-induced atrophy, TNFα binds to its receptor, activating the IκB kinase (IKK) that phosphorylates IκB, which in turn translocates NF-κB to the nucleus, where it can alter the transcription of several genes, i.e., can induce MuRF1 activation, promoting protein degradation (figure 4). However, under disuse-induced atrophy, the NF-κB pathway is activated by another mechanism other than TNFα.

Transgenic mice overexpressing IKKβ display acute muscle wasting likely to have been caused by the activation of IKKβ/NF-κB/MuRF1. Although genetic inhibition of IKKβ/NF-κB does not show an overt phenotype, upon denervation muscle atrophy was reduced. Therefore, NF-κB activation is sufficient to induce muscle atrophy in vivo and block the NF-κB pathway can ameliorate the atrophy but only partially, thus suggesting other pathways contributing to the muscle atrophy, i.e., a cross-talk between NF-κB and the Akt pathway has been proposed since TNFα can also produce insulin resistance and inhibit the IGF1/Akt pathway. Paradoxically, mechanical stretch can activate both pathways, speculating that mechanical stretch activates Akt, which in turn activates IKK/NF-κB. Moreover, oxidative stress can also activate NF-κB, leading to skeletal muscle protein loss.
Figure 4. Simplified scheme of the major signalling pathways controlling fibre size. Red lines indicate inhibition and green lines activation.
Mitogen-activated protein kinases (MAPKs) pathway
ERK1/2, JNK, and p38 MAPK cascades are part of the MAPK pathway. Growth factors mainly activate ERK1/2 cascade whereas stress stimuli, such as oxidative stress, pro-inflammatory cytokines, osmotic shock, and stretch, are mainly responsible for activating the JNK cascade and p38 MAPK pathway. Rats subjected to immobilization show an up-regulation of p38 and JNK. Following activation, MAPKs can either phosphorylate different cytoplasmic targets or translocate to the nucleus and directly or indirectly influence transcription. JNK is a negative regulator of the insulin-dependent Akt pathway during disuse atrophy. Activation of IRS is inhibited by inflammatory cytokines, such as TNFα, via JNK. Furthermore, p38 MAPK is another trigger for the up-regulation of atrogin-1 independently of Akt, and TNFα can activate atrogin-1 mRNA expression via p38 MAPK. The mechanosensitive TGFβ and Ras pathways converge on the MAPK pathway, inducing constitutive activation. This is of specific interest since a strong activation of the TGFβ/MAPK by stress stimuli has been reported in patients with AQM, leading to muscle actin-cytoskeleton reorganization, atrophy, apoptosis, and proteolysis. Moreover, the α7β1 integrin, linking laminin in the extracellular matrix with actin in the cytoskeleton, has been shown to be a negative regulator of MAPK phosphorylation.

Dystrophin-glycoprotein complex (DGC)
DGC is formed of several proteins: dystrophin, dystroglycans, sarcoglycans, sarcospan, syntrophins, neuronal nitric oxide synthase (nNOS) and dystrobrevin. In the past, it was thought that DGC has only a structural role, linking the cytoskeleton and the sarcolemma, but today it is known that DGC plays an essential role for the muscle membrane integrity and mediating intracellular signals between the cytoskeleton, membrane, and extracellular matrix. Mutations in DGC genes or in DGC-associated molecules, such as caveolin-3, cause muscular dystrophies, e.g., Duchenne muscular dystrophy. One study on cancer-induced muscle atrophy in transgenic mice found membrane abnormalities correlated with a loss of DGC integrity and a significant decrease of dystrophin expression under atrophic conditions; moreover, muscle wasting became more apparent in tumour-induced mice lacking dystrophin. In the same study, transgenic mice overexpressing dystrophin showed less muscle loss and a significant down-regulation of atrogin-1 and MuRF1 under atrophic conditions, concluding that dystrophin expression is sufficient to prevent muscle loss. Since it has been suggested that disruption of DGC can inhibit Akt activation, it is speculated that dystrophin may activate Akt, which in turn blocks FoxO activation and the down-regulation of atrogenes, although other mechanisms that are Akt-independent should be considered. During disuse-
induced atrophy, DGC is disrupted and nNOS is translocated to the cytoplasm, generating nitric oxide (NO) that activates FoxO3, up-regulating the atrogenes independently of NF-κB pathway (figure 4)\textsuperscript{162}.

**Myostatin**

Myostatin (also known as growth and differentiation factor 8 (GDF8)) is a powerful negative regulator of skeletal muscle growth and it is a member of the transforming growth factor-beta (TGF-β) super family. Myostatin inhibits satellite cell activation, differentiation and muscle development by down-regulating Pax7, MyoD and myogenin, and by up-regulating the expression of some cell cycle inhibitors such as p21 and p53\textsuperscript{163-165}. Mutations in the myostatin gene in multiple species cause a marked increase of muscle mass\textsuperscript{166} and male transgenic mice overexpressing myostatin show decreased muscle mass with 20% less CSA and reduced myonuclei number\textsuperscript{167}. During embryogenesis, myostatin promotes the muscle mass increasing fibre number (hyperplasia) and increasing fibre size (hypertrophy), but in adulthood this growth is restricted to the increase of muscle fibre size\textsuperscript{163}. Contrary to the original idea, it seems that hypertrophy caused by myostatin inhibition is independent of satellite cells activation in adult muscles\textsuperscript{168}. Besides increasing muscle mass in myostatin deficient animals, increased muscle force was not detected\textsuperscript{169}. Myostatin mRNA expression is up-regulated by hind-limb unloading, denervation, hypoxia and food deprivation, contributing to muscle atrophy\textsuperscript{170-173}, however, in critically ill patients and in septic rats, it is down-regulated\textsuperscript{174,175}. Glucocorticoids, which are elevated in muscle wasting conditions, can increase myostatin secretion\textsuperscript{116}. Regarding the effects of exercise on myostatin levels\textsuperscript{116}, the results are not consistent, suggesting a time-, intensity-dependent effect.

Myostatin binds to the activin receptor IIB (ActRIIB), a type II TGF-β receptor, which in turn activates the type I serine/threonine kinase receptors, activin receptor-like kinase 4/5 (ALK4/5), resulting in the phosphorylation and activation of the transcription factors Smad2 and Smad3 that form then a complex with Smad4. This complex is translocated to the nucleus, altering the transcription of target genes and inducing muscle atrophy\textsuperscript{176-178} (figure 4). If myostatin-induced atrophy is caused by an increase of the rate of proteolysis and/or a decrease of protein synthesis remains still unclear, but it seems that protein synthesis is more affected than degradation\textsuperscript{177}.

A crosstalk between the Akt and myostatin pathway has been suggested since myostatin can block the Akt/mTORC1 signalling pathway, inhibiting myoblast differentiation and myotube hypertrophy\textsuperscript{178}. A feed-forward mechanism has been proposed: myostatin activates Smad2, which inhibits Akt/mTORC1 that leads to myostatin’s activation of Smad2\textsuperscript{176}. Furthermore, inhibition of the Smad2/3 stimulates muscle hypertrophy independent of
satellite cells but partially dependent of mTOR signalling\textsuperscript{176}. In any case, Akt seems to be dominant over the TGF-\beta pathway since high Akt activity prevents the atrophy caused by Smad2/3 activation\textsuperscript{176}; furthermore, the addition of IGF-1 can reactivate the Akt pathway, reverting the effects of myostatin on either myoblast or myotubes\textsuperscript{178}. Myostatin can also activate p38 MAPK independently of Smad activation\textsuperscript{179}. Moreover, myostatin also plays a role in glucose metabolism, increasing glycolysis in skeletal muscle cells through AMPK\textsuperscript{180}.

The blocking of myostatin signalling has been widely studied as a possible therapeutic strategy for patients with muscle atrophy, using specific inhibitors and genetic manipulations, e.g., inhibiting ActRIIB, the myostatin receptor\textsuperscript{181}. There are other proteins that inactivate myostatin by direct binding such as growth and differentiation factor associated protein 1 and 2 (GASP-1/-2), Latent TGF-\beta Binding Protein-3 (LTBP-3), follistatin, follistatin-like 3 (FSTL-3) and decorin\textsuperscript{166}. Caveolin-3, a scaffolding protein, can also inhibit myostatin signalling, binding to the type I receptors, blocking their activation and, consequently, suppressing Smad2 activation\textsuperscript{182}. In addition, myostatin activates the inhibitory Smad7 that interferes in the Smad complex, blocking myostatin signalling by a negative feedback loop\textsuperscript{183}.

**JunB**

It has recently been demonstrated that the transcription factor JunB plays a critical role in the regulation of muscle mass, being necessary for the maintenance of muscle size and promoting hypertrophy, independently of the Akt/mTOR pathway and satellite cell activation\textsuperscript{184}. Moreover, overexpression of JunB in denervated muscles prevents atrophy by interacting with FoxO3 and inhibiting its binding to MuRF1 and Atrogin-1 promoters. In addition, JunB overexpression is able to dephosphorylate Smad3, leading to the inhibition of myostatin expression and hence, contributing to muscle growth\textsuperscript{184}. JunB is a downstream effector of signal transducer and activator of transcription-3 (STAT3), a member of JAK/STAT signalling pathway. STAT3 is activated by cytokines and growth factors including interleukin-6 (IL6), IGF-1 and fibroblast growth factor (FGF), through different stimuli, e.g., exercise\textsuperscript{185}. At the transcriptional level, JunB mRNA expression increases in response to insulin and acute exercise\textsuperscript{186,187}, and decreases in atrophy induced by fasting or denervation\textsuperscript{188,189}. Interestingly, earlier studies in denervated muscles have shown an up-regulation of JunB mRNA expression\textsuperscript{190,191}.  

Aims of the present investigation

The aim of this thesis is to achieve a better understanding of the cellular and molecular mechanisms underlying the muscle wasting and weakness in ICU patients with AQM, and evaluate specific therapeutic strategies and efficient rehabilitation programs. In accordance with the stated objectives, a rodent ICU model was used to address the mechanistic and therapeutic aspects of the disease. Subsequently, the knowledge obtained from this model was translated into a clinical intervention study.

Specific aims:

1. Determine the mechanisms underlying the preferential myosin loss and impaired skeletal muscle structure and function associated with AQM (papers I and IV).

2. Describe the time course of changes in skeletal muscle morphology and function as well as in protein turnover in response to the ICU intervention (paper I) and mobilization strategies (paper III).

3. Elucidate time-resolved gene expression patterns in an experimental rat ICU model (paper I) subjected to unilateral passive mechanical loading (paper III).

4. Determine the effects of passive mechanical loading on muscle wasting and function in either an experimental rat ICU model (paper III) or ICU patients (paper IV).
Materials and Methods

Animals (papers I, II and III)
Female Sprague-Dawley rats were anaesthetized (isoflurane), mechanically ventilated, monitored and pharmacologically paralysed with α-cobratoxin for durations varying from 6h to 14 days. The sham-operated control animals were anesthetized (isoflurane), spontaneously breathing, given intra-arterial and intra-venous solutions, and sacrificed within two hours after the initial anaesthesia and surgery, but they were not pharmacologically paralyzed with α-cobratoxin.

ICU patients (paper IV)
A total of seven (four females and three males, aged 56-67 years) mechanically ventilated ICU patients were included in the study. Patients anticipated to require mechanical ventilation for 10 consecutive days or longer were recruited. Patients had typically been exposed to mechanical ventilation and immobilization for 0-3 days (1.7 ± 0.9 days) prior to initiating the intervention and monitoring. Patients with a previous history of neuromuscular disease were not included in the study. No evidence based guidelines for severe sepsis were found in these patients. None of them received systemic administration of NMBA and only one patient received administration of inhaled corticosteroids. Propofol was intravenously administered in all patients.

Mechanical loading (papers III and IV)
In the paper III, the left leg of the animal was activated for 6 hours at the shortest duration and 12 hours per day at durations 12 hours and longer throughout the experiment, using a mechanical lever arm that produced continuous passive maximal ankle joint flexions/extensions at a speed of 13.3 cycles per minute.
In the paper IV, the left leg of the ICU patients was subjected to passive mechanical loading for 2.5 hours, 4 times per day during 7-11 days, i.e., continuous passive anatomically correct motion from 30° plantar flexion to 25° dorsiflexion were generated at a speed corresponding to 150° per minute.

Ultrasound measurements (paper IV)
The tibialis anterior (TA) CSAs of the ICU patients were measured every day during the intervention period (7-11 days) using a real-time ultrasound scanner. Scans were taken transversally on relaxed muscles at three locations: 50, 40 and 30% of the distance from the proximal part of the fibula head to the distal tip of the lateral malleolus. The captured muscle images were stored, the region of interest (TA muscle mass without bone and fascia) was manually selected and CSA was measured. CSA mean was calculated as the mean of three consecutive measurements at the three different locations (50, 40 and 30%) on each leg.

Electrophysiological measurements (paper IV)
To investigate the properties of the nerve-muscle interaction, electrophysiology was performed. Motor (n. fibularis, and tibialis) and sensory nerve (n. suralis and fibularis superficialis) were measured bilaterally using surface electrode both for stimulation and recording. Studies were performed on the first and final day of the observation period in all patients. At the final day of the period, concentric needle EMG was performed in the m. vastus lateralis and tibialis anterior bilaterally.

Muscle biopsy and muscle fibre membrane permeabilization (papers I, II, III and IV)
In the paper IV, muscle samples were obtained from both the loaded and unloaded TA muscle and when specified, from the vastus lateralis, using the percutaneous conchotome method at the final day of the observation period. In the papers I, II and III, the TA, plantaris, EDL, gastrocnemius and soleus muscles were dissected from the loaded left leg and the unloaded right leg immediately after euthanasia. One half of the soleus, TA and EDL muscles together with plantaris and gastrocnemius muscles were quickly frozen in liquid nitrogen cooled by liquid nitrogen and stored at −160 °C for further analyses. The other halves of the soleus, EDL and TA muscles were immediately placed in an ice-cold relaxing solution (in mmol/l: 100 KCl, 20
imidazole, 7 MgCl₂, 2 EGTA, 4 ATP, pH 7.0; 4 °C). Small bundles of ∼25–50 fibres were dissected free from the muscle and tied with surgical silk to glass capillary tubes at slightly stretched lengths. The bundles were then placed in a skinning solution (relaxing solution containing glycerol, 50:50 vol/vol) at 4°C for 24 h, after which they were transferred to −20 °C. All the bundles were cryo-protected within one-two weeks after skinning and subsequently snap frozen in liquid propane cooled by liquid nitrogen and stored at −160 °C for further studies in single muscle fibre.

Contractile measurements of single muscle fibres (papers I, III and IV)

On the day of the experiment, a fibre segment 1 to 2 mm long was left exposed to the experimental solution between connectors leading to a force transducer and a lever arm system. The apparatus was mounted on the stage of an inverted microscope. While the fibre segments were in relaxing solution, the sarcomere length was set to 2.65-2.75 µm by adjusting the overall segment length. The diameter of the fibre segment between the connectors was measured through the microscope. Fibre depth was measured by recording the vertical displacement of the microscope nosepiece while focusing on the top and bottom surfaces of the fibre. The focusing control of the microscope was used as a micrometer. Fibre CSA was calculated from the diameter and depth, assuming an elliptical circumference, and was corrected for the 20% swelling that is known to occur during skinning. Maximum velocity of unloaded shortening (V₀) was measured by the slack-test procedure. Maximum active tension (P₀) was calculated as the difference between the total tension in the activating solution (pCa 4.5) and the resting tension measured in the same segment while in the relaxing solution. All contractile measurements were carried out at 15 °C. Specific tension (ST) was calculated as maximum tension (P₀) normalised to CSA. In the paper I, stiffness and relative force–pCa and stiffness–pCa relationships were also studied. After the mechanical measurements, each fibre was placed in urea buffer in a plastic micro centrifuge tube and stored at -80 °C for subsequent electrophoretic analyses.

Enzyme-histochemistry and immunocytochemistry (papers I, II and IV)

To measure the CSA of muscle fibres, enzyme histochemistry was performed. Cross-sections (10µm) were cut perpendicular to the longitudinal axis of muscle fibres with a cryostat at -20 °C. The sections were stained for myofibrillar ATPase after alkaline and acid pre-incubations (paper I) and
NADH (paper IV). CSAs, roundness and the smaller diameter were measured in a total of 50-100 muscle fibres in the central region of the biopsy cross-section using an inverted microscope and an imaging software.

To detect the presence of a specific protein and its localization in the muscle fibre, immunocytochemistry was used. Cryo-sections (10 μm) of muscle biopsy were cut. Expression and subcellular localisation of MuRF-1/2, p62/SQSTM1, serum response factor (SRF) (paper I), cleaved caspase-3, MyHC type I (paper II), nNOS (paper IV) and laminin (paper II and IV), were performed. All samples were stained with identical primary and secondary antibody dilutions and immunofluorescence was analysed by confocal microscopy.

**Myosin heavy chain isoform expression (papers I, III and IV)**

MyHC isoform expression was determined by 6% SDS-PAGE. The total acrylamide and bis concentrations were 4% (w/v) in the stacking gel and 6% in the running gel, and the gel matrix included 30% glycerol. The gels were stained, scanned and the volume integration function was used to determine the fibre type proportion.

**Myosin, actin and total protein quantification (papers I, III and IV)**

Total protein content was determined from 10 μm muscle cross-sections dissolved in 100 μl 8 M urea buffer after centrifugation and heating (90°C for 2 min), using the NanoOrange Protein Quantification Kit (Invitrogen, Carlsbad, CA, USA) in papers I and III, or the Pierce® 660 Protein assay (Thermo Fisher Scientific Inc, Rockford, IL, USA) in the paper IV, according to manufacturer’s instructions. The fluorescence of the samples was measured and related to a standard curve.

Actin and myosin quantification was determined by 12% SDS-PAGE. The acrylamide concentration was 4% (w/v) in the stacking gel and 12% in the running gel, and the gel matrix included 10% glycerol. The gels were stained and subsequently scanned in a soft laser densitometer. The volume integration function was used to quantify the amount of myosin and actin.
Western Blot (papers I and III)

To identify and quantify the expression of specific proteins, western blots were running. In the paper I, HSP70, αβ-crystallin, atrogin-1, calpain-1, LC3B and MuRF1 expression were measured in EDL. In the paper III, Troponin (Tn) isoform expression was measured in TA and Grp94 protein levels were quantified in the plantaris muscle. The immunoblots were scanned in a soft laser densitometer and the signal intensities were quantified using the volume integration function and normalised to actin content.

Protein oxidation detection (paper III)

To assess the formation of protein carbonyl groups, the OxyBlot protein oxidation detection kit was used according to the manufacturer’s detailed protocol. Levels of oxidated protein were quantified using the NIH ImageJ analysis software and normalized to the densitometric value of the Ponceau red staining of the corresponding actin band.

Post-translational modifications (paper IV)

Mass spectrometric peptide sequencing and analysis was applied to study post-translational modifications of myosin (for details, see paper IV).

Fractional protein synthesis rate (papers I and III)

Fractional protein synthesis rate (FSR) was used to measure the rate of muscle protein synthesis in the superficial and deep part of the gastrocnemius using [ring-$^{13}$C$_6$]phenylalanine as tracer (for details, see papers I and III).

Total RNA isolation and quantification (papers I, II, III and IV)

Total RNA was extracted from frozen muscle tissue (10-30mg) using a QiagenRNAeasy® Mini Kit (Qiagen, Inc., Valencia, CA). RNA-concentrations were then quantified using the fluorescent nucleic acid stain, Ribogreen® (Molecular Probes, Eugene, OR), on a fluorescence spectrophotometer.
Quantitative RT-PCR (papers I, II and IV)

qRT-PCR was used to quantify the mRNA levels for MyHCs isoforms, skeletal α-actin, the myosin binding proteins, as well as MAFbx/atroglin-1, MuRF1, calpain-1 and Map1lc3b. RNA purification, cDNA synthesis and qRT-PCR analysis was performed as it is described in papers I, II, and IV.

Gene expression profiling (papers II and III)

Three micrograms of total RNA from the proximal gastrocnemius muscle samples were extracted and processed to generate biotin-labeled cRNA. Each sample was then hybridized to Affymetrix Rat Gene 1.0 ST Array. Microarray data were background-adjusted, normalized and log-transformed summarized values. In order to search for the differentially expressed genes between the samples from the different days an empirical Bayes moderated t-test was applied. A linear model was fitted to the data, control vs. unloaded (paper II) and unloaded vs. loaded (paper III) at the following time durations: 6h-4 days, 5-8 days, and 9-14 days. To address the problem with multiple testing, the p-values were adjusted according to Benjamini and Hochberg. Probe sets with a minimum fold change of ±2 and adjusted p-value < 0.05 (paper II) or a minimum fold change of ±1.5 (paper III) at least in one time point were included in further analyses.

Statistics (papers I, II, III and IV)

SigmaPlot software (Systat Software, Inc., CA, USA) was used to generate descriptive statistics. Means, standard errors of the means (SEM) and linear regression analysis were calculated according to standard procedures. Paired t-test was used in pairwise comparisons between unloaded and loaded leg. One- and two-way analyses of variance (ANOVA) and the Tukey post-hoc test were used when comparing multiple groups. When the normality test failed, a one way ANOVA on ranks, i.e., Kruskal–Wallis, and the Dunn's post-hoc were performed. Differences were considered significant at p < 0.05.
Results and Discussion

Signs of AQM are seen from the fifth day of ICU intervention (paper I)

A unique experimental rat ICU model where animals were mechanically ventilated, sedated, pharmacologically paralysed with NMBAs, and extensively monitored between 6 h and 14 days was used. This model has several similarities with modern intensive care treatment, such as muscle unloading, mechanical ventilation, sedation and blocked neuromuscular transmission during an extended period of time, but without the confounding influence of differences in systemic disease and pharmacological treatment. Results showed that, from the fifth day of the ICU intervention, a phenotype resembling the features of AQM observed in ICU patients was developed. These features included: 1) marked atrophy with progressive muscle weight loss, and a decrease of single muscle fibre contractile properties, i.e., CSA, specific force (maximum force normalized to muscle fibre CSA, (SF)) and stiffness, and 2) preferential myosin loss, a factor typical of AQM patients. Previous studies using a porcine experimental model with exposure to different combinations of NMBAs, CS, sepsis, muscle unloading and mechanical ventilation, did not detect any significant changes in myofibrillar proteins, muscle fibre mass or force generating capacity during the 5-day experiment, in agreement with our results. In a similar manner, ICU patients showed atrophy after only 4 days of intervention, also in accordance with our observations.

Mechanical silencing is a key factor triggering AQM (papers I and IV)

Sepsis, NMBAs, CS, and immobilization have all been postulated as the major causative agents for the development of AQM; primary disease, organ transplantation, prolonged mechanical ventilation, multi-organ failure, and hyperglycemia may also be risk factors for this myopathy. However, there are discrepancies in the literature as to the triggering factors underlying this condition; furthermore, AQM could be developed in the absence of N MBA and/or CS, and independently of underlying disease, thus it still remains unclear as to the fundamental cause. Therefore, it is hypothesised that common components of the ICU intervention per se, such
as immobilization, mechanical ventilation and sedatives, play a crucial role in the development of AQM.

We demonstrated that in either, the experimental rat ICU model or ICU patients subjected to immobilization, mechanical ventilation, and sedation, mechanical silencing (absence of external strain related to weight bearing and internal strain caused by myosin-actin activation during contraction due to pharmacological paralysis or sedation) is underlying the muscle wasting, impaired muscle function and the preferential loss of myosin, which are the most recognizable characteristics of AQM. Therefore, the mechanical silencing associated with the ICU intervention is proposed as a dominant factor triggering AQM, while sepsis, NMBAs, CS, and other factors certainly contribute to aggravate its prognosis.

Protein turnover underlying ICU intervention (Papers I and II)

In order to improve our understanding of the mechanisms underlying the muscle wasting and weakness associated with the ICU intervention and the time course of the protein turnover, the experimental rat model was used. There are three main proteolytic systems that work as partners during muscle proteolysis: UPS, ALS and the calpain system. The UPS is responsible for 80-90% of protein degradation. Atrogin-1 and MuRF1 are muscle-specific ubiquitin ligases; since they are up-regulated in many atrophy models, they are considered markers of atrophy. MuRF1 ubiquitinates thick filament proteins, which later will be degraded by the proteosome.

Results showed that under ICU intervention, there was a specific temporal pattern of proteolytic pathways with an early and maintained up-regulation of the atrogenes, MuRF1 and atrogin-1, from 6 hours to 8 days and a slight decrease from 9 to 14 days, followed by an activation of the ALS, calpain system and endoplasmic reticulum-associated degradation (ERAD) from 5 to 14 days at the transcriptional level. A dramatic down-regulation of genes coding a large number of sarcomeric proteins, including myosin, was observed from 5 to 14 days. At the protein level, MuRF1 was up-regulated at early durations (6h to 14 days), while Atrogin-1 expression was not significantly increased during the observation period. LC3b and calpain-1, taken as markers of the autophagy-lysosome and calpain systems respectively, increased their expression from 9 to 14 days. The loss of myosin was significant from 5 to 14 days.

MuRF1 and MuRF3 localize to the Z-disc whereas MuRF2 binds near to the C-terminus of titin in the M-band region, however, under stress MuRF1/2 can shift to the nucleus. In the rat ICU model, MuRF1/2 and their ligand p62, were translocated from the cytoplasm to the nucleus where they were co-localized with serum response factor (SRF) after 4 days of the ICU
condition. Nuclear MuRF1/2 can alter the transcription of several genes \(^{12}\), e.g., can repress the transcription of MyHC and actin \(^{12,196,197}\). After 9 days, MuRF1/2, p62 and SRF were localized in the cytoplasm, in the perinuclear space. Therefore, MuRFs do not only control the degradation of thick filaments, but are also involved in transcriptional regulation of contractile proteins.

Taken together, these results suggest that muscle wasting and preferential loss of myosin were preceded by activation of the UPS. The subsequent activation of the ALS may contribute to muscle protein degradation or else has a protective role since ALS is very important for muscle tissue homeostasis and muscle fibre survival \(^{136,198}\). Myofibrillar protein degradation products leave severe cytotoxic effects \(^{199}\) on muscle cells and ALS may protect myofibres by sequestering these toxic products.

Several chaperones involved in protein folding, assembly and protection against protein degradation and acute stress were up-regulated from 5 to 14 days due to the ICU intervention at the gene level. The proteins Hsp70 and \(\alpha\beta\)-crystallin were up-regulated from 9 to 14 days. Hsp70 overexpression protects against fatigue induced by muscle injury \(^{200}\) and in disuse conditions it prevents muscle atrophy \(^{201}\). \(\alpha\beta\)-crystallin regulates desmin assembly and folding \(^{132}\) and protects actin against degradation under oxidative stress \(^{202}\); moreover, mice lacking of \(\alpha\beta\)-crystallin died prematurely with extensive muscle wasting \(^{203}\). Therefore, the up-regulation of chaperones/Hsp may prevent excessive muscle atrophy.

Regarding protein synthesis, genes that promote protein synthesis (translational initiation and elongation factors and ribosomal proteins) were up-regulated from 5 to 14 days, contrary to other atrophy models, e.g., hind-limb unloading for 14 days \(^{204}\). The fractional protein synthesis rate increased from 9 to 14 days. A stimulation of muscle protein synthesis signalling pathways has been observed in ICU patients \(^{175}\), supporting our findings. Therefore, the mechanical silencing associated with the ICU intervention seems to have a different effect compared with other atrophic conditions, promoting protein synthesis at longer durations. However, the marked atrophy seen in the rat ICU model and in ICU patients indicates that protein degradation is exceeding the synthesis rate. The increased protein synthesis may be caused by an increase of available amino acids resulting from the increased proteolysis and/or an attempt to restore muscle mass \(^{175}\).
Temporal gene expression profile in an experimental rat ICU model (paper 2)

The results from this study showed:

1. A specific temporal pattern of genes that code protein degradation pathways with an early up-regulation of MuRF1 and atrogin-1, followed by an activation of the ALS, ERAD and calpain system from 5 to 14 days, in parallel with a dramatic down-regulation of genes that code a large number of sarcomeric proteins, including myosin, and up-regulation of heat shock proteins/chaperones and genes involved in protein synthesis machinery (see discussion above).

2. Activation of oxidative stress, cell cycle arrest and pro-apoptotic genes mainly from 5 to 14 days. Muscle atrophy conditions induce oxidative stress increasing ROS that activate apoptotic and proteolytic pathways\textsuperscript{205}. In order to cope with oxidative stress, antioxidant genes were up-regulated. Several cell cycle regulators, especially those that promote cell cycle arrest, were up-regulated during the ICU intervention, which may lead to apoptosis\textsuperscript{117}.

3. Activation of the caspase cascade from 9 to 14 days. This study showed that myonuclear apoptosis, as measured by the presence of cleaved caspase-3, was markedly elevated after 9-14 days of the ICU treatment. Atrophy activates apoptotic pathways in order to lose individual myonuclei from muscle fibres, however, this theory is still not clear since in disuse atrophy, no loss of nuclei has been reported\textsuperscript{206,207}. Furthermore, the role of apoptosis differs between different models of atrophy\textsuperscript{143}. Muscle atrophy was significant after 5 days while a significant increase in apoptotic nuclei was observed first after 9-14 days, therefore it is evident that the atrophy precedes the apoptosis. Thus, apoptosis was not a mediator of the muscle atrophy observed in response to the ICU intervention, at least not during the first two weeks.

4. Altered expression of genes involved in the regulation of muscle size from 5 to 14 days. Genes that participate in the NF-κB pathway were activated from 5 to 14 days in parallel with the decrease of FoxO1 expression, suggesting that NF-κB may maintain the activation of MuRF1 after 5 days, leading to skeletal muscle protein degradation\textsuperscript{147}. Moreover, some genes involved in the preservation and promotion of muscle mass were up-regulated while genes that inhibit muscle growth were down-regulated (see discussion below).
ICU muscle wasting may induce compensatory mechanisms to diminish the muscle atrophy (paper II)

In spite of the fact that some genes that stimulate muscle development and growth were down-regulated, it is worthwhile pointing out that myogenic factors (Myogenin, Myf5 and Myf6) and hepatocyte growth factor (Hgf) involved in the activation of quiescent satellite cells and new muscle fibre formation during embryogenesis and also in adult muscles\(^4\), were all up-regulated, especially from 5 to 14 days.

Moreover, myostatin mRNA expression, the powerful negative regulator of muscle growth, was down-regulated from 5 to 14 days, in parallel with the up-regulation of its inhibitor, follistatin, and the down-regulation of the myostatin receptors, suggesting a promotion of muscle growth independently of satellite cells activation\(^{168,208}\). Myostatin mRNA expression has been reported to be up-regulated in several atrophy models\(^{170-173}\); however, it has been found to be down-regulated in critically ill patients after an average of 6 days in ICU\(^{173}\) and in septic rats\(^{174}\). Therefore, the muscle wasting associated with the ICU intervention induces changes in myostatin expression not seen in other atrophy models. Myostatin can also activate p38 MAPK\(^{179}\), being a possible explanation to the down-regulation of some genes that participate in p38 MAPK pathway observed in this study.

In addition, JunB mRNA expression was up-regulated after 9-14 days. JunB is necessary for the preservation of muscle mass and is able to promote hypertrophy and protein synthesis independently of the Akt/mTOR pathway and satellite cells. Overexpression of JunB preserves muscle atrophy, reducing MuRF1 and atrogin-1 activity and inhibiting myostatin signalling\(^{184}\).

The up-regulation of Runx1 mRNA expression was also observed from 5 to 14 days. Runx1 is strongly expressed after denervation, and plays an important role in the maintenance of muscle mass, preventing denervated myofibres from excessive autophagy\(^{209}\).

Therefore, the activation of some important genes involved in the maintenance of muscle mass and stimulation of muscle growth, together with the down-regulation of myostatin that inhibits muscle growth and the activation of several genes that participate in protein synthesis and skeletal muscle fibre regeneration from 5 to 14 days, may indicate somehow compensatory mechanisms to reduce the excessive sarcomeric degradation and muscle wasting seen under ICU condition.
Mechanical silencing induces specific myosin post-translational modifications (paper IV)

Mass-spectrometry analysis revealed specific myosin post-translational modifications (PTMs) in immobilized, sedated and mechanically ventilated ICU patients absent in healthy controls. Almost all the new modifications were located deep in the motor domain of the myosin, a region less prone to oxidative modifications due to its structure and the presence of hydrophobic domains; therefore, PTMs in the myosin motor domain may be a sign of acute oxidative stress. We speculated that neuronal nitric oxide synthase (nNOS) plays a role in these modifications as three out of the five new PTMs were deamidations and it is known that nitrite anions can induce the deamidation of proteins. nNOS generates nitric oxide (NO) that produces peroxynitrite (ONO₂⁻), a reactive oxidant and nitrating agent that is tightly regulated under physiological conditions but under acute oxidative stress has detrimental effects. Results showed an increase of nNOS levels (as an indicator of NO activity) and a shift from the sarcolemma to the cytoplasm. This dislocation has also been reported during muscle atrophy caused by hind-limb suspension or amyotrophic lateral sclerosis, demonstrating that cytoplasmic nNOS produces NO and up-regulates MuRF1 and atrogin-1 via FoxO3. In conclusion, nNOS has arisen as a key factor underlying the specific myosin PTMs induced by mechanical silencing. These new PTMs may affect the regulation of contraction and lead to muscle proteolysis.

Beneficial effects of passive mechanical loading on skeletal muscle (papers III and IV)

Unilateral passive mechanical loading applied 12 hours per day in ICU rats significantly attenuated the loss of muscle mass and specific force associated with the ICU intervention in both fast- and slow-twitch muscles, resulting in ~44% higher single fibre CSA and double force generating capacity in loaded vs. unloaded after 9-14 days. Several studies have also found that static stretching suppresses the muscle wasting associated with cast immobilization, denervation and hind limb suspension, which is in accordance with our results. The sparing of muscle mass and function was paralleled with the transcriptional up-regulation of contractile proteins from 5 to 14 days, leading to a reduced myosin loss from 9 to 14 days, although in the fast-twitch EDL muscle the reduction was not statistically significant probably due to the fact that protein turnover is slower in fast than slow muscles. Moreover, passive loading induced an up-regulation of genes involved in sensing stress signals and muscle growth such as Ankrd2 and the myostatin inhibitors, caveolin-3 and follistatin, after 5 days. In addition, passive loading was able to relieve the increased fractional protein synthesis rate observed in the ICU rats after 9-14 days.
Unilateral passive mechanical loading applied 2.5 hours, 4 times per day during 9±1 days had also positive effects on muscle function in deeply sedated and mechanically ventilated ICU patients, although it was not sufficient to counteract the loss of muscle mass. These discrepant results between the experimental model and the clinical study may be explained by the influence of underlying disease in the ICU patients, species- and age-related differences (rats were young while the age of patients ranged between 56 and 67 years), the delayed monitoring of muscle mass in ICU patients due to delays in obtaining signed informed consents, or by the fact that the ICU patients were not exposed to NMBAs.

Given that passive loading caused an increase of SF in both the animal model and the ICU patients in spite of a lower than normal myosin:actin ratio, we hypothesized that loading may induce MyHC PTMs, altering its function in muscle contraction. However, no specific MyHC PTMs were found in ICU patients associated to the passive loading effect. Other PTMs as well as modifications of other sarcomeric proteins, such as actin, MyLC, or tropomyosin, may be affected by the loading condition, leading to an impaired contractile response and this needs to be addressed in future studies.

The beneficial effect of passive mechanical loading on alleviating the muscle atrophy in sedated, pharmacologically paralyzed and mechanical ventilated rats together with the improvement in muscle function seen in both the experimental rat model and the ICU patients support the importance of early physical mobility therapy in deeply sedated and mechanically ventilated ICU patients, resulting in shortening of ventilator days and ICU stay, as well as reducing the financial costs and enhancing patient prognosis and QoL of survivors.

**Passive mechanical loading can alleviate the oxidative stress associated with mechanical silencing (paper III)**

Oxidative stress is an imbalance between oxidant and antioxidant levels, and it is commonly induced by disuse atrophy and aging that increase ROS levels. ROS mediate the activation of NF-κB, MAPKs and FoxO, and can also cause mitochondrial dysfunction, leading to increased protein breakdown, decreased protein synthesis and apoptosis. Carbonylation is a common marker of oxidative stress since it is an irreversible reaction and carbonyls are quite stable. Protein carbonylation levels are seen to be increased due to the ICU intervention in the experimental rat model from 9 to 14 days, paralleled by an increased oxidative stress response (heat shock proteins and antioxidants) at the gene level from 5 to 14 days (paper II). However, on the loaded side, carbonylation levels remained stable and there was a decrease of genes involved in oxidative stress response. In addition,
the oxidative stress response was also examined by quantifying GRP94 protein content, a calcium sensitive chaperone that regulates calcium homeostasis in the endoplasmic reticulum (ER). GRP94 overexpression inhibits the apoptosis induced by ischemia and ER-stress and protects against oxidative stress in myogenic cells\textsuperscript{229-231}. GRP94 was highly increased in the loaded side from 5-8 days compared with the unloaded side, thus it is likely that it plays a role in the lower oxidative stress seen in the loaded side. These results indicate a protective role of passive loading against the oxidative stress induced by the mechanical silencing associated with the ICU intervention.
Conclusions

This thesis has investigated the mechanisms underlying the muscle wasting and weakness in ICU patients with AQM and shown that the ICU condition in itself, i.e., immobilization, sedation and mechanical ventilation, induces a phenotype resembling the severe muscle wasting and/or paralysis associated with AQM, i.e., preferential myosin loss, muscle atrophy, and a dramatic decrease in muscle fibre force generating capacity. The complete mechanical silencing of skeletal muscle, i.e., absence of external strain (weight bearing) and internal strain (myosin-actin activation) due to the pharmacological paralysis or sedation associated with the ICU intervention, is the primary trigger of AQM. Moreover, the mechanical silencing induces specific PTMs in the motor domain of myosin that may influence the regulation of contraction and cause muscle proteolysis in immobilized and sedated ICU patients. The higher nNOS expression found in the ICU patients and its cytoplasmic translocation are forwarded as a probable mechanism underlying these modifications.

Temporal gene expression patterns have been uncovered in response to the ICU intervention: an early and maintained up-regulation (6h-14d) of MuRF1 and atrogin-1, followed by an up-regulation of the proteolytic systems, antioxidant genes and genes that participate in cell cycle arrest and apoptosis (5-14d). At the longest duration (9-14d), genes involved in immune response and the caspase cascade were up-regulated. Genes coding contractile, regulatory, and structural sarcomeric proteins were down-regulated (5-14d). The activation of some genes that promote muscle development and growth (increase of myogenic factors and JunB, and down-regulation of myostatin) together with the up-regulation of genes that regulate the protein synthesis machinery (5-14d) suggests a protective mechanism to compensate for the excessive muscle proteolysis and wasting in response to the ICU intervention.

Passive mechanical loading induces significant positive effect on muscle function in ICU patients with AQM and is able to attenuate the oxidative stress associated with the mechanical silencing. Whether it can prevent the muscle wasting accompanying the ICU condition remains uncertain since passive loading could alleviate the muscle mass loss in the experimental ICU model, but it was not able to do so in the immobilized, sedated, and
mechanically ventilated ICU patients. The beneficial effects of passive loading on muscle structure and function strongly support a shift in physical therapy among immobilized ICU patients towards a very early intervention strategy.
Acknowledgments

This work was carried out at the Clinical Neurophysiology, Department of Neuroscience, Uppsala University Hospital, Uppsala, Sweden in collaboration with research groups at Department of Chemistry, BMC, Uppsala University, Uppsala, Sweden; Hershey Medical Center, Hershey, USA; the Mayo Clinic College of Medicine, Rochester, USA; King’s College London BHF Centre for Research Excellence, London, UK; Division of Endocrinology, Wayne State University, Detroit, USA, and at Department of Sciences, Padova University, Padova, Italy.

I would like to thank many people who directly or indirectly contributed to the completion of this thesis. I would specially like to express my sincere gratitude to:

Professor Lars Larsson, my supervisor, for introducing me to the muscle research and transmitting your passion and knowledge to me. I will always be grateful for your trust and for pushing me to aim high.

Dr. Julien Ochala, for your patience and answers when I came suddenly into your office and said: I have a question. You have a great gift to make difficult things easy!.

My boys: Meishan, Sudhakar, Nicola, Rizwan, Guillaume and Johan. It was fantastic to share these years with you all. I cannot imagine better colleagues than you. I learned a lot from each one of you and I take with me many good memories and stories. Thanks a lot for everything!.

The new girls: Rebeca, Hannah and Marit. I am so sorry for not having more time to share with you all and you have met me “under-stress”. I wish you the best with your studies and Carpe diem ☼. Hannah, thanks a lot for taking your time to proof-read, spell and grammar check this thesis.

Magnus Andersson. I really enjoyed working with you in the NIVA.

Ann-Sofie and Ivette, the best lab assistants around the world as well as very good and cheerful people.
Inger Hedlund, for not only your assistance with the administrative work but also for taking care of us.

All staff at Clinical Neurophysiology. You have created the nicest work atmosphere. This department is a role model in all aspects. I am so grateful to each of you for your warm welcome, hospitality and help. I will miss your smiles, Friday breakfasts, our talks and dancing the Macarena in St. Lucia 🎉.

All the patients, their relatives and volunteers for their collaboration and kind donation of muscle biopsies. I truly hope that the outcome of our research can someday help other people in similar situations.

My friends in Spain, Germany, and Sweden for supporting me all these years. I am really honoured with your friendship 😊.

A mis padres, por confiar en mí y en mis posibilidades y por enseñarme a luchar por lo que uno quiere y no rendirse jamás.

A Tita y Tino, por estar siempre ahí, en lo bueno y en lo malo a pesar de la distancia.

A cada uno de los miembros de la familia Solís-Hernández, por traerme la luz, el calor y la alegría cuando lo necesitaba.

A Olga, no tengo suficientes palabras para agradecerte todo lo que has hecho. Gracias por tu ayuda, apoyo, consejos y cuidados. ¡Contagias alegría de vivir!.

A Víctor. Gracias por compartir tu vida conmigo. Llenas y equilibras cada uno de mis días “here, there and everywhere”💍. A tu lado, soy mejor.

Mónica Llano-Díez
April 22^{nd}, 2012
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Acta Universitatis Upsaliensis

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