CHAPTER 1  INTRODUCTION
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1. INTRODUCTION

1.1 Muscle weakness and weaning failure at the ICU

Patients with critical illness develop substantial skeletal muscle weakness and physical disability under the influence of disuse, systemic inflammation, medications and electrolyte disturbances. There is a strong association between acquired muscle weakness and length of stay at the Intensive Care Unit (ICU) and morbidity. For ICU survivors muscle weakness is the most prominent long-term complication as it leads to functional impairment that can last for years. In particular, weakness of the diaphragm – the skeletal muscle that accounts for 70% of muscular effort needed for respiration– is precarious. Diaphragm dysfunction leads to complications and prolongation of ICU and hospital stay as it is strongly correlated with prolonged dependency on mechanical ventilation, i.e. weaning failure.

The incidence of weaning failure is high. It occurs in approximately 25% of patients on mechanical ventilation and the consequences are large: approximately 40% of the total time on mechanical ventilation is spent on the process of weaning. Weaning failure is associated with post-extubation distress, morbidity and long term functional limitations after hospital discharge. In recent years, the focus on developing therapies to combat diaphragm weakening and weaning failure in critically ill patients has expanded, but the nature of diaphragm weakness and its underlying pathophysiological mechanisms are poorly understood.

In the section ‘Respiration and the diaphragm’ (1.2) we explain the basics of physiology of respiration and the role of the diaphragm. First, the organ systems involved in respiration are discussed. To explain how force development of the respiratory muscles is controlled we zoom in from neural to molecular level. The section ‘The pathophysiological mechanisms of diaphragm weakness’ (1.3) explains how skeletal muscle weakness develops, and the subsequent section ‘Risk factors for diaphragm weakness in critically ill patients’ (1.4) explains what factors may increase the risk to develop develop diaphragm weakness at the ICU. Subsequently, it is briefly discussed how diaphragm function can be determined in ‘Determination of diaphragm function in patients’ (1.5). The introduction concludes with the section ‘Aims and methods’ (1.6) and the section ‘Outline of the thesis’ (1.7).

1.2 Respiration and the diaphragm

Each living system is dependent on the exchange of environmental oxygen for the metabolite carbon dioxide. In mammals carbon dioxide is transported by the blood in its dissolved form, bound to hemoglobin at red blood cells or as bicarbonate to the
INTRODUCTION

capillaries that surround the alveoli of the lungs. Here, the affinity of hemoglobin for oxygen exceeds the affinity for carbon dioxide, resulting in their exchange. Oxygen rich blood leaves the alveoli and is transported to all tissues of the body. With every respiratory cycle the air in the alveoli is replaced by new air, a process that is enabled by contraction of the respiratory muscles. On average in rest, every minute the lungs receive 5 liters of blood and 4 liters of air. The alveoli are tiny gas filled structures surrounded by a net of capillaries, creating a maximal surface for gas exchange. One pair of lungs contains approximately 300 million alveoli, creating a surface the size of half a tennis court.

Contraction of the intercostal muscles causes an outward movement of the thorax and contraction of the diaphragm causes a downward expansion of the thoracic cavity towards the abdomen, see insert in Figure 1. The enlargement of the thorax automatically leads to an enlargement of the lungs, as they are connected to the thoracic wall due to sub-atmospheric pressure. Enlargement of the lungs leads to a decrease in intra-thoracic pressure, a relation described by Boyle’s law. As the pressure in the lungs becomes smaller than the atmospheric pressure, air flows into the lungs; see Figure 2. When the pressure in the lungs equals that of atmospheric pressure, the respiratory cycle reverses. The inspiratory muscles relax and compliance of the lungs automatically resizes the lungs back to their resting volumes. The pressure in the lungs exceeds the atmospheric pressure and air is forced to leave the respiratory system (Figure 2C). (Note that for this section descriptions from Vander’s Human Physiology: The Mechanisms of Body Function were used).

Figure 1 A simplified overview of the main respiratory muscles, the lungs and the pleura of the lungs.
Figure 2. (A) Contraction of the respiratory muscles enlarges lung volume. (B) As atmospheric pressure becomes larger than pressure in the lungs, air flows into the lungs. (C) Relaxation of the respiratory muscles and elastic recoil of the lungs restore the lung to their resting volume, thereby increasing pressure in the lungs to exceed the atmospheric pressure and thus air is forced to leave the lungs. Thick black line denotes diaphragm, dark grey open arrows denote movement of air, closed light grey arrows denote movement of the lungs. (D) Illustration of Boyle’s Law. Drawings by Pleun Hooijman.

**Neural control of diaphragm activation**

Contraction of the diaphragm muscle can be controlled consciously via the somatic nervous system, however, it is predominately controlled involuntary by rhythmical and automatic activation via the autonomic nerve system. At the lower half of the brain stem, in the medulla oblongata, axons of the inspiratory center are rhythmically excited under control of the expiratory center. The upper part of the brain stem, the pons, regulates the medulla; its apneustic center stimulates the inspiratory neurons of the expiratory and inspiratory centers to control depth and speed of inspiration, and the pneumotaxic centers transmit inhibitory signals to the inspiratory center to control duration of inspiration. Also chemoreceptors –structures that sense changes in pO$_2$, pCO$_2$ and pH– and mechanoreceptors –structures that sense stretch in the airways– regulate respiration. (Note that for this section descriptions from Murray and Nadels Text book of respiratory medicine. 5th edition were used).

Thus, under the control of reflexes, rhythmical excitation of the axons of the inspiratory centers induce excitation of the phrenic nerves that have their axons spread out to the left and right spheres of the diaphragm muscle.

**From excitation of the phrenic nerves to the release of calcium**

When the action potential that travels across the phrenic nerves reaches its synaptic end, acetylcholine is released from the axon terminal and binds to receptors at the motor end-plate of the diaphragm muscle. This binding elicits an end-plate potential.
which initiates rapid depolarization of the sarcolemma; see insert in Figure 3B. The wave of positive charges promotes the opening of the voltage-gated sodium channels at the sarcolemma, inducing an influx of sodium into the sarcoplasm, resulting in membrane depolarization. The depolarization wave spreads across the sarcolemma, including the T-tubules that surround each myofibril. Here, it activates the L-type calcium channels, which on their turn induce the opening of the ryanodine receptors (alias calcium release channels) of the sarcoplasmic reticulum (SR), resulting in calcium release. Repolarization of the membrane occurs when the sodium channels become inactivated, the potassium channels open and potassium fluxes outwards. When the sarcolemma is completely repolarized, the potassium channels close and the sodium/potassium pump actively restores the concentration gradients of potassium and sodium. (Note that for this section descriptions from Cell Physiology: Source Book Sperelakis 12).

**Figure 3** Simplified overview of activation and architecture of diaphragm muscle fibers. (A) Under control of the respiratory centers of the lower half of the brain stem, an action potential is spread across the phrenic nerves from the cervical segments towards the ends at the diaphragm. (B) Nerve-end at one diaphragm muscle fiber. The end-plate potential triggers sodium and potassium currents across the sarcolemma, that subsequently results in release of calcium from the sarcoplasmic reticulum and diffusion of calcium towards the contractile filaments. (C) Schematic representation of the architecture of the contractile machinery. Straight lines denote magnifications. Drawings by Pleuni Hooijman.

**From calcium release to formation of cross bridges**
The next step is the conversion of biochemical energy into mechanical energy. The calcium released from the SR diffuses through the sarcoplasm and reaches the contractile machinery 13. The contractile machinery consists of many proteins and protein complexes that in concert with the rise of calcium and available cytosolic ATP,
propel the conversion of chemical energy into mechanical energy, in order to do work, i.e. movement. Within the centre of the contractile machinery thick and the thin filaments are present; see Figure 3C. The thick filament consists of dimers of y-shaped myosin molecules that via a head region project towards the thin filament. These head domains powered by the hydrolysis of ATP, project towards the thin filament in order to generate force and perform work. The thin filament consists mainly of double stranded helices of actin monomers and dimers of tropomyosin, which together with troponin compose the functional unit for muscle contraction. Troponin consists of the protein subunits troponin T that binds to tropomyosin, troponin I that via tropomyosin interaction inhibits actin-myosin formations, and troponin C that binds calcium released from the SR, which promotes the movement of tropomyosin away from the myosin-binding sites located on actin.

The actin-myosin cross bridge
In presence of calcium, the myosin-binding sites on actin are available for cross bridge formation (Figure 3C). As indicated above, ATP hydrolysis powers myosin interaction with actin, resulting in the formation of a weak-binding complex, dominated by the formation of the myosin-ADP-Pi complex. After the release of Pi, the myosin head undergoes a conformational change that promotes the strongly-bound attachment of the myosin head (i.e. myosin-ADP complex), culminating in the pulling of the actin strand towards the middle of the sarcomere, i.e. power stroke. Depending on the muscle load, the muscle fiber shortens (concentric contraction), preserves its length (isometric contraction) or elongates its length (eccentric contraction). After the power stroke, the actin-myosin complex is in a low-energy state, dominated by the presence of the myosin-ADP state. As soon as the ADP diffuses away from the myosin head, a new ATP molecule is able to bind to myosin and promote its dissociation from actin. Hydrolysis of ATP re-starts in the detached myosin head, ‘refueling’ its high-energy confirmation, and a new cross bridge can be formed. The calcium is actively transferred back from the cytosol to the SR by sarco-endoplasmic reticulum Ca^{2+}-ATPase.

Energy source of the muscle fiber
Cross bridge cycling and calcium pumping account for the majority of ATP consumption in the muscle fiber. Aerobic production of ATP takes place at mitochondrial level. Briefly, mitochondria consist of an outer phospholipid bilayer membrane that surrounds the intermembrane space and the inner membrane; see Figure 4. The inner membrane is both an insulator and a chemical barrier that complexes into folded structures called cristae. At the inner membrane mitochondrial complexes enable the formation of a concentration gradient of protons that powers the generation of ATP during oxidative phosphorylation by ATP synthase (complex V).
**INTRODUCTION**

![Schematic illustration of a mitochondrion and the electron transport chain where oxidative phosphorylation takes place.](image)

**Figure 4** Schematic illustration of a mitochondrion and the electron transport chain where oxidative phosphorylation takes place. $Q=$ubiquinol, $C=$cytochrome C, $Cr=$ creatine phosphate, $Cr\sim P=$creatine phosphate, $Pi=$inorganic phosphate, $ANT=$ adenine nucleotide translocase (antiport), I, II, III, IV and V= electron transport complexes. Drawings by Pleuni Hooijman.

**Glycolysis, citric acid cycle and oxidative phosphorylation**

During oxidative phosphorylation, electrons released from the oxidation (i.e. loss of electrons) of substrates are transferred via a series of protein complexes at the inner membrane of the mitochondria. These biochemical reactions contribute to an energetic (hydrogen) potential, which is essential to drive the reconversion of ADP to ATP. The starting point for the oxidation of these substrates takes place with glycolysis\(^{18,19}\) in the sarcoplasm, whereby one glucose molecule is hydrolyzed to two molecules of pyruvate. Inside the mitochondrial matrix, these pyruvate molecules are decarboxylated by pyruvate dehydrogenase to acetyl-CoA, which is subsequently used in the citric acid cycle. In the citric acid cycle electrons from acetyl-CoA and from oxidized fatty acids are removed and used to convert NAD\(^+\) and FAD\(^{2+}\) to NADH and FADH\(_2\), respectively. Subsequently, NADH binds to Complex I (NADH dehydrogenase), thereby donating one electron. As the electrons travel through the complex, protons move from the matrix towards the intermembrane. The electrons are transferred to a ubiquinone molecule in the inner membrane. At complex III (cytochrome bc1 complex) oxidation of one molecule of ubiquinone results in the reduction of two molecules of cytochrome c that induces another flux of protons into the matrix. At complex IV (cytochrome c oxidase), oxidized cytochrome C delivers two electrons that react with oxygen resulting in another addition of protons to the proton potential. Complex II (fumarate reductase) is both used in the citric acid cycle and for oxidative phosphorylation. Complex II oxidizes succinate to fumarate, a reaction that catalyzes the conversion of FADH\(_2\) to FAD, adding two electrons to ubiquinone but no proton transfer over the inner membrane. Finally, at complex V (ATP-synthase), the proton gradient is used for the production of ATP
from ADP and Pi. The newly formed ATP either diffuses out of the mitochondria or as creatine phosphate via the mitochondrial creatine kinase shuttle.

To summarize, the diaphragm is the main inspiratory muscle that is activated by excitation of the phrenic nerves. Spreading of a bioelectrical signal across the sarcolemma of diaphragm muscle fibers activates a biochemical reaction that results in the biomechanical movement of the diaphragm muscle. This causes enlargement of the alveolar space, enabling a flow of air from the environment into the lungs. These processes consume ATP that is mainly produced by mitochondrial oxidative phosphorylation. In the next section, the mechanisms that regulate diaphragm muscle are discussed.

1.3 Pathophysiological mechanisms of diaphragm weakness

Skeletal muscles are able to adapt and respond to (patho)physiological stimuli. Such adaptations are observed at several places throughout the nerve-motor unit system; from the cerebral cortex to the cross bridge. Increased activation of cellular degradation and proteolytic pathways, and decreased protein synthesis lead to a net negative balance of protein turnover, and thus for example loss of muscle fibers. Next we discuss the ubiquitin-proteasome pathway, calpains/caspases and the autophagy-lysosome pathways that are important interacting pathways that regulate muscle fiber plasticity and protein turnover; see figure 5.

Dismantling of proteins of the contractile filaments is facilitated by caspases and/or calpains with release of myosin and actin from the sarcomeres; see figure 5A. Caspases are enzymes that hydrolyze peptide bonds in a reaction that depends on cysteine residues in the caspase active site. They are present in the cell as inactive pro-enzymes that require dimerization and often cleavage before activation. Caspases play essential roles in apoptosis, necrosis, and inflammation. Calpains are cysteine proteases that cleave proteins by a nucleophile attack at the peptide bond. The calpain and caspase systems both influence and support another.

In the ubiquitin-proteasome system, proteins are targeted for degradation by the 26S proteasome through covalent attachment of a chain of ubiquitin molecules; see Figure 5B. The E3 ligases Muscle ring finger-1 (MuRF-1) and atrogin-1 (MAFbx) are considered key markers of proteolytic activity that are specific for skeletal muscle. These E3 ligases bind protein substrates and catalyze the movement of the ubiquitin from the E2 enzyme to the substrate. This process is repeated until a chain of at least four ubiquitin molecules attaches to the protein, which subsequently allows recognition by the 26S proteasome and cleavage into short peptide by its catalytic core. Activation of the ubiquitin-proteasome pathway is controlled by the Protein kinase B (Akt) - Forkhead box O (FoxO) transcription factors pathways. Under healthy conditions, insulin growth factor (IGF) phosphorylates Akt that induces
phosphorylation of FoxO to inhibit nuclear transport. In a disease state, Akt is not activated and unphosphorylated FoxO shuttles to the nucleus, inducing muscle atrophy MAFbx and MuRF-1 gene expression, that promotes muscle atrophy.

Another important homeostatic mechanism in skeletal muscle is the autophagy-lysosome pathway 28. The autophagy system generates double membrane vesicles that engulf portions of cytoplasm, organelles, glycogen and unfolded and toxic proteins. Autophagosomes are then delivered to lysosomes for degradation of their contents 29,30, see figure 5C.

Also the mitochondria are involved in the regulation of catabolic pathways. Fragmentation of mitochondria activates the FoxO3 via AMP-activated protein kinase AMPK, which is essential in the mediation of energy-consuming and producing pathways, and is associated with loss of muscle mass 27,31–34. Mitochondria are an important source of ROS that may directly - or indirectly via mitochondrial damage - induce activation of AMPK and other atrophic pathways 35,36. Notably, mitochondria are able to release pro-apoptotic factors such as cytochrome c 37. Thus, mitochondria may play a key role in the regulation of muscle atrophy. In the past years it has become evident that mitochondria also have a crucial role in cell signaling and homeostasis.

Figure 5 Pathways of muscle fiber degradation (A) Distmantling of contractile proteins by caspases/calpains, (B) the ubiquitin-proteasome pathway, (C) autophagy and (D) ROS production and release of cytochromes from mitochondria. Drawings by Pleuni Hooijman.

1.4 Risk factors for diaphragm weakness in critically ill patients
The pathophysiological mechanisms mentioned in the previous section are stimulated by several factors that are commonly present in critically ill patients on mechanical ventilation and may cause muscle atrophy and weakness of the diaphragm.

One of those stimuli is muscle disuse \(^{38-43}\). Compared to unsupported ventilation, the activity levels of the diaphragm are strongly reduced during mechanical ventilation. Whether and how much the diaphragm remains active during respiratory support depends on the ventilatory mode and its settings \(^{44-46}\). Since the diaphragm is a muscle that never rests (during non-supported respiration), it may be very sensitive to reduced levels of activity. Therefore, it has been suggested frequently that disuse may be one of the main contributors to ventilator induced diaphragm dysfunction \(^{47}\).

In addition, critically ill patients may have elevated levels of catabolic inflammatory mediators that affect diaphragm muscle strength. For example tumor necrosis factor-\(\alpha\) \(^{48,49}\) and other catabolic cytokines can be released into the circulation due to ventilation-induced lung injury \(^{49}\) or other diseased organs. In septic patients, who suffer from systemic inflammatory response syndrome and an infection, proinflammatory cytokines and free-radicals are markedly increased, and caspase and calpain are activated, which lead to a negative protein turnover \(^{50}\). The changes arising from sepsis may influence excitation-contraction coupling via decreasing membrane excitability, alter calcium homeostasis and other subcellular sites, contributing to diaphragm weakness \(^{50,51}\).

Critically ill patients may also suffer from malnutrition \(^{52}\) and receive moderate doses of corticosteroids and other medication known to affect diaphragm function \(^{53,54}\).

Thus critically ill patients are exposed to a range of factors that may lead to diaphragm dysfunction. However, it remains unknown whether diaphragm dysfunction truly occurs these patients.

### 1.5 Determination of diaphragm function in patients

There are several ways to determine diaphragm function. For example, transdiaphragm pressure can be determined \textit{in vivo} by measuring the pressure differences in the esophagus and stomach while activating the phrenic nerves by magnetic stimulation. Animal models consistently indicate that \textit{in vivo} transdiaphragm pressure is reduced with 40-50\% during mechanical ventilation \(^{41,55,56}\). Also in mechanically ventilated critically ill patients, diaphragm strength has been investigated using non-invasive measurements; ultrasound revealed reduced motion and thinning of the diaphragm \(^{57-59}\), and by magnetic stimulation of the phrenic nerves a reduced capacity to generate pressure was observed \(^{60-63}\). However, these pressure measurements are an indirect way to determine diaphragm function. To elucidate muscle function at muscle fiber level and to unravel the pathological mechanism underlying dysfunction, muscle tissue...
INTRODUCTION

is required. In animal models, it was found that diaphragm strips and single fibers display rapid atrophy and a 30-50% reduction in maximal contractility after mechanical ventilation. Since technical limitations make it difficult to obtain such material from critically ill patients ex vivo, most investigators have used muscle samples from brain-dead organ donors, who received mechanical ventilation prior to organ harvest. These studies revealed severe atrophy in diaphragm muscle fibers, which was not observed in non respiratory muscles such as the pectoralis major muscle. Based on these observations, it was suggested that changes at the level of individual diaphragm fibers may play a critical role in the development of diaphragm weakness in critically ill patients. However, up to date, there is lack of evidence that these findings are truly present in critically ill patients, and the pathophysiological mechanisms are far from understood.

1.6 Aims and methods

At present, the nature of the diaphragm weakness and its underlying pathophysiological mechanisms are poorly understood. Establishing whether in critically ill patients the individual diaphragm muscle fibers exhibit contractile weakness, atrophy and mitochondrial dysfunction will provide rationale for treatment strategies that specifically improve the contractility of diaphragm fibers to facilitate weaning (van Hees et al., 2009; Doorduin et al., 2012). Investigation of diaphragm muscle biopsies is required to elucidate the nature of diaphragm weakness in critically ill patients since they allow ways to study muscle fiber compartments, structure and size, and, importantly, they enable the determination of the contractile properties of individual diaphragm fibers.

In this thesis the following hypotheses were tested: (1) that diaphragm muscle fibers of mechanically ventilated critically ill patients display atrophy and contractile weakness, (2) that activation of the ubiquitin-proteasome pathway plays a critical role in the development of atrophy of the diaphragm in mechanical ventilated critically ill patients, and (3) that contractile strength is recovered by a fast-skeletal troponin activator. Finally, (4) we studied morphological and functional changes in mitochondria of diaphragm muscle fibers of mechanically ventilated critically ill patients.

To test our hypotheses, we obtained biopsies of the diaphragm and of non-inspiratory muscles of mechanically ventilated patients prior to elective or emergency surgery, and compared these biopsies with those obtained from patients undergoing elective resection of an early lung malignancy (controls). We determined the size and the contractile strength of individual muscle fibers, and evaluated components of the proteolytic ubiquitin-proteasome pathway. Additionally, we exposed the fibers to a novel fast-troponin activator CK-2066260. Finally, we studied the key proteins for metabolic regulation, mitochondrial network dynamics and content and function of protein complexes.
1.7 Outline of the thesis

In chapter 2, the fiber cross sectional area and contractility of diaphragm muscle fibers of mechanically ventilated brain dead organ donors are studied.

In the subsequent chapters extensive analyses on diaphragm biopsies from critically ill ICU patients that received mechanical ventilation prior to surgery are described. In chapter 3, the changes in fiber cross sectional area, contractility and the underlying pathophysiological pathways that contribute to these changes were determined in diaphragm and non-inspiratory muscle biopsies. In chapter 4, we determined whether a fast-skeletal troponin activator, CK-2066260, would improve diaphragm fiber force of mechanically ventilated diaphragm muscle fibers by improving their sensitivity for calcium. In chapter 5, we studied the key proteins for metabolic regulation, mitochondrial network dynamics and content and function of complexes protein.

In chapter 6, the main conclusions of this thesis are given, followed by future perspectives.

In chapter 7, a summary of the main findings is presented in English and in Dutch.