CHAPTER 6 SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

6. SUMMARY, DISCUSSION AND FUTURE PERSPECTIVES

Summary and discussion

In this thesis, we compared diaphragm muscle biopsies of brain dead organ donors and mechanically ventilated critically ill patients with those of control patients who had elective lung surgery. The investigation of diaphragm muscle biopsies is indispensable to elucidate the nature of diaphragm weakness in critically ill patients. We determined the cross sectional area and the contractile strength and cross bridge cycling kinetics of individual muscle fibers. In addition, we evaluated components and regulators of the ubiquitin-proteasome pathway, one of the major proteolytic pathways. We tested the potential of the fast-troponin activator CK-2066260 to improve muscle fiber contractility. Finally, we measured mitochondrial fragmentation and complexes that regulate the activation of atrophic pathways and affect ATP production.

Chapter 1 provides a general overview of the physiology of respiration. It explains that the diaphragm is the main inspiratory muscle. Spreading of an action potential across the sarcolemma of muscle fibers induces calcium release and cross bridge cycling and thus to the development of force. Contraction of diaphragm fibers enlarges the lungs and pulls air from the environment into the alveoli. Mechanical ventilation is frequently used in the Intensive Care Unit as a life-saving therapy for respiratory failure. However, several studies on animal models and humans have indicated that diaphragm dysfunction develops during mechanical ventilation. Diaphragm weakness contributes to a delay in weaning from mechanical ventilation, and to an increased duration of hospital stay and morbidity in patients admitted to the Intensive Care unit [1,5–7].

Findings of reduced motion and thinning of the diaphragm [5958] and a reduced capacity to generate transdiaphragm pressure [63] [51] suggest that diaphragm dysfunction develops in critically ill patients. Elucidating the changes that occur at the level of the individual muscle fibers is important for understanding the pathophysiology of diaphragm dysfunction but has hardly been carried out so far. This is clearly due to the technical challenges to obtain diaphragm biopsies from critically ill patients. An alternative to biopsies in critically ill patients, is acquiring diaphragm tissue of brain dead organ donors since they receive controlled mechanical ventilation prior to organ harvest. Several studies indicated atrophy and activation of proteolytic pathways in diaphragm fibers of these donors [62,67–69], but no studies have reported the effects of mechanical ventilation on muscle fiber function. Therefore, in **chapter 2**, we compared tissue from brain-dead organ donors (n=9) with control patients (n=9) that had surgery for resection of a suspected early lung malignancy. Our findings revealed that both slow-twitch and fast-twitch diaphragm muscle fibers of brain dead organ donors did not differ from controls with respect to (1) fiber cross-sectional area, (2) muscle fiber contractility expressed as absolute and specific force, (3) the calcium sensitivity of force generation and (4) kinetics of cross-bridge cycling, and also that (5) myosin heavy chain

content was not affected. The apparent discrepancy between the findings from previous work that reported a significant reduction of diaphragm muscle fiber cross sectional area and our study might be a result of the duration of mechanical ventilation *after* diagnosis of brain death, which was relatively short in the present study (26h) compared to the duration reported in those earlier studies (34h in Levine et al [68] and 80h in Jaber et al [62]. Other explanations may include differences in the duration and the modes of mechanical ventilation *prior* to the diagnosis of brain death since various modes of mechanical ventilation are known to differentially impact the diaphragm [44,46,63], or differences in the exposure to compounds that affect muscle contractile function, such as corticosteroids [162]. Unfortunately, such information prior to the diagnosis of brain death is usually unavailable. Thus, the best explanation is that 26h of controlled mechanical ventilation is not sufficient to elicit diaphragm fiber atrophy and dysfunction in humans.

It is crucial to study diaphragms of critical ill patients in order to understand weaning failure since brain dead organ donors do not exhibit the clinical features of critically ill patients. Consequently, it is unknown whether findings from brain dead organ donors translate to critically ill patients. In **chapter 3**, we described studies of diaphragm muscle biopsies from 22 critically ill patients who received mechanical ventilation prior to surgery and from 14 control patients who underwent surgerical resection of a suspected early lung malignancy. We observed that (1) critically ill patients had approximately 25% smaller slow-twitch and fast-twitch fiber cross sectional area compared to controls, and that (2) absolute maximal force of these fibers was more than 50% lower. This reduction in force persisted after normalization to cross sectional area, which denotes reduced maximal tension. The reduction in maximal tension was mainly due to a reduction in the number of attached cross bridges during activation. Our findings also revealed that diaphragms of critically ill patients (3) have significantly higher ubiquitination of proteins and higher content of striated muscle-specific ubiquitin E3 ligases MuRF-1 and MAFbx – that facilitate the conjugation of ubiquitin to proteins[23]. The key role of MuRF-1 in development of muscle weakness was underlined by our proof-of-concept study performed in MuRF-1 knockout mice, showing that (4) these mice were protected against the development of diaphragm contractile weakness during mechanical ventilation. The observed reduction in the contractile force of diaphragm fibers is comparable to the reduction in diaphragm strength in vivo, as determined previously by phrenic nerve pacing [51,63]. This indicates that changes at the diaphragm fiber level may largely account for the in vivo diaphragm weakness in critically ill patients. This study provides rationale for the development of treatment strategies like blockers of muscle specific ligases of the ubiquitin-proteasome pathway to prevent atrophy or therapies that target the contractility of diaphragm fibers to improve weaning from mechanical ventilation.

Fast skeletal troponin activators are a novel class of small molecule drugs that improve the contractility of skeletal muscle fibers. One of these activators, called *tirasemtiv* (formerly CK-2017357)[81], is currently under study in patients with amyotrophic lateral sclerosis [NCT01709149]. In **chapter 4** we tested the ability of the fast skeletal troponin activator CK-2066260 – an analogue of *tirasemtiv* - to improve the contractile strength of diaphragm muscle fibers. We obtained diaphragm biopsy specimens from critically ill patients (n=10) and compared them with control patients undergoing elective lung surgery (n=10). We observed that (1) the negative logarithm of the calcium concentration needed to obtain 50% of maximal force the pCa_{50} (i.e. myofilament calcium sensitivity) was significantly lower in fast-twitch diaphragm fibers critically ill patients. In a representative subset of control (n=3) and critically ill patients (n=4) we exposed single diaphragm fibers to 5μM of CK-2066260. Our findings further revealed that compared to vehicle, this (2) significantly increased the calcium sensitivity of diaphragm fibers in both patient groups. Importantly, we found that (3) at physiological calcium levels CK-2066260 restored the contractile force of fast-twitch diaphragm fibers of critically ill patients back to levels observed in untreated fibers from controls. Thus, CK-2066260 significantly augments the *ex vivo* contractile strength of diaphragm muscle fibers. Future studies should test the efficacy of these drugs in improving success of weaning in critically ill patients.

Our results in chapter 3 and 4 suggest that diaphragm weakening in critically ill patients can be largely accounted for by contractile weakness and atrophy of individual diaphragm fibers and that the ubiquitin-proteasome pathway plays a key role in proteolysis of diaphragm muscle protein. It remains incompletely understood, however, which mechanisms trigger proteolytic pathways in diaphragm fibers of critically ill patients. Recent findings indicated that the AMPK-FoxO3 signaling axis is a major regulator of expression of atrophic pathways like the ubiquitin-proteasome pathway [32–34] and that mitochondrial fission/fusion are signaling events that facilitate its activation [27,31,32]. Another important role of mitochondrial fission and fusion is that they enable efficient mixing of the matrix and inner membrane and facilitates the exchange of constituents for optimal ATP production [138]. If the mitochondria fail to produce ATP during reloading of the diaphragm when respiratory support is discontinued, they may impair weaning of mechanical ventilation of critically ill patients. In **chapter 5** we compared the morphology and function of mitochondria of mechanically ventilated critically ill patients (n=28) with control patients (n=27). We observed (1) an increase of mitochondrial fission proteins and a decrease in mitochondrial fusion proteins, (2) a decrease in the mitochondrial biogenesis regulator PGC1-α, (3) activation of the energy stress sensor AMPK, (4) a decrease in electron transfer protein complexes III, IV and V, but (5) preservation of complex I and II content and respiration with considerable heterogeneity among patients. Damaged mitochondria and mitochondrial networks may contribute to muscle atrophy, limit

efficient production of ATP and increase production of ROS. We postulate that mitochondrial dysfunction impairs diaphragm function, which may be especially important during weaning.

Future perspectives

It is frequently debated what the main causes are of difficult weaning of mechanical ventilation in the ICU [6,47,74,125,163,164]. If one knows its main contributors, treatment strategies may be adapted and new therapies may be developed. Only animal models allow control of all experimental conditions, but the translation of their findings to ICU patients is limited. In the patients studied in the present thesis, mechanical ventilation is the common denominator, and diaphragm disuse caused by mechanical ventilation may explain, at least partially, our findings of diaphragm weakness, ubiquitin-proteasome activation and mitochondrial adaptations. However, in addition to mechanical ventilation, in these patients there are several other interacting and confounding phenomena associated with critical illness. For instance, critically ill patients are typically subject to general muscle disuse [38–43], malnutrition [52], inflammation [49] and sepsis [50,51]. Critically ill patients also often receive moderate doses of corticosteroids and other medication that may affect diaphragm function [53,54]. How does one elucidate whether, and to which extent, these contributors affect the structure and contractility of muscle fibers of critically ill patients?

In our small study population, we did not observe a correlation between the duration of mechanical ventilation, presence of sepsis, daily nutritional intake on one side, and the size or contractility of single fibers on the other side. However, this does not imply that such correlation does not exist. We have the impression that in our patient group, which was of limited size (n=28), such correlations are extremely difficult to establish due to the high heterogeneity of the patients. Each critically ill patient has its unique history of disease, reason for admission to the ICU, and progresses differently during ICU stay. Furthermore, the applied duration and mode of mechanical ventilation may vary per patient, and within each patient this can vary over time. To identify specific disease entities that are associated with the observed diaphragm fiber weakness a larger patient cohort of patients is required.

Ideally, changes in the diaphragm are studied by repeated measurements in patients. One of the few studies with repeated measurements on diaphragm function in critically ill patients was performed by Hermans et al [63]. They showed that the duration of mechanical ventilation is associated with a logarithmic decline in transdiaphragmatic pressure during magnetic stimulation of the phrenic nerves. Using sonography, Grosu et al also studied diaphragm muscle daily from the day of intubation until the patient underwent extubation or tracheostomy or died, and they found 6% thinning of diaphragm per day on mechanical ventilation [59]. To improve our understanding of the

changes occurring at the level of the individual diaphragm muscle fibers, serial biopsies in time are required.

Wollersheim et al. succeeded to obtain serial biopsies from m. quadriceps in critically ill patients [106]. Their findings revealed significant upregulation of MuRF-1 and MAFbx five days after ICU admission, a time point at which atrophy had not developed yet, whereas after fifteen days the E3-ligase levels were back to control levels but fiber atrophy had started to develop. Performing such study with serial biopsies from the diaphragm is extremely challenging, however maybe not impossible, since some critically ill patients undergo repeated laparotomies or repeated thoracotomies. It would be worthwhile the challenge to obtain such serial diaphragm biopsies.

To conclude, the population of critically ill patients studied in this thesis is a valuable reflection of the typical mix of patients admitted to a medical-surgical ICU. A larger group of such patients - or ideally serial diaphragm biopsies of these patients - would improve our understanding of the development of diaphragm weakness and would aid in identifying treatment targets.

Although there are limitations in clinical extrapolation, we have collected unique diaphragm biopsy tissue that helps to answer many research questions. Our findings of activation of the ubiquitin proteasome system, increased fission and decreased fusion of mitochondria, the activation of AMPK and a fall in levels of PGC-1α enable to elucidate their relation. For instance, we may study whether and how AMPK-sensed energy stress influences catabolic pathways in the diaphragm, whether mitochondria indeed undergo fission to help to maintain bioenergetics capacity and to eliminate of damaged mitochondria by autophagy. Future studies may also elucidate whether diaphragm fibers fail to produce ATP efficiently – which may partially explain failure to wean – and what the role is of fragmented mitochondria. In future analyses of mitochondria we could make a distinction between sarcolemmal and intermyofibrillar mitochondria, which would provide a more specific insight into mitochondrial changes. Finally, while it has been amply demonstrated that oxidative stress is required for the activation of catabolic systems in mechanically ventilated animals, this is not clear in humans. It would be worthwhile to investigate the expression of key enzymes of antioxidant defense systems (SOD, catalase) and oxidative damage (protein oxidation level) in the diaphragm biopsies.

What should be the next steps in studying potential treatment strategies?

Our findings indicate that exposure to fast skeletal troponin activators of isolated diaphragm muscle fibers improved the contractility of sarcomeres. Doorduin et al. recently reported that infusion of the calcium sensitizer Levosimendan improved contractile function and neuro-mechanical efficiency of the diaphragm in healthy controls [71]. Hence, we suggest that administration of compounds that improve the

sensitivity for calcium might be a more powerful therapeutic approach to improve diaphragm fiber strength and neuro-mechanical efficiency in critically ill patients. Future studies should test the efficacy of these types of drugs in improving weaning in critically ill patients. Secondly, our findings of preserved diaphragm strength in MuRF-1 deficient mice during mechanical ventilation strengthen the idea that slowing down of proteolysis by targeting ubiquitin-ligases might be a successful therapeutic approach to facilitate weaning. Proteasome inhibitors already have been successfully used to block atrophy in various animal models [116–120] but may affect other cell systems as well. Thus, there is a need for compounds that block E3 ligases specifically. Our group plans to perform experiments to test whether such compounds prevent diaphragm weakening in mechanically ventilated mice.

Conclusion

Our findings of severe diaphragm atrophy, contractile weakness and alterations of mitochondria in diaphragm fibers of critically ill patients may largely explain weakness of the diaphragm impairing weaning from mechanical ventilation, which is frequently observed in clinical practice. Our innovative approach to harvest diaphragm tissue provides ways to test potential therapeutic strategies and to take a step forward in the fight against weaning failure.