Intensive care unit-acquired weakness

Pathophysiology and diagnosis

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INTENSIVE CARE UNIT-ACQUIRED WEAKNESS

PATHOPHYSIOLOGY & DIAGNOSIS

ESTHER WITTEVEEN

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pathophysiology and diagnosis

Esther Witteveen

Intensive care unit-acquired weakness: pathophysiology and diagnosis
Thesis, University of Amsterdam, the Netherlands

INTENSIVE CARE UNIT-ACQUIRED WEAKNESS:

PATHOPHYSIOLOGY AND DIAGNOSIS

ACADEMISCH PROEFSCHRIFT
ter verkrijging van de graad van doctor
aan de Universiteit van Amsterdam
op gezag van de Rector Magnificus
prof. dr. ir. K.I.J. Maex
ten overstaan van een door het College voor Promoties ingestelde commissie,
in het openbaar te verdedigen in de Agnietenkapel
op woensdag 29 november 2017, te 14.00 uur
door Esther Witteveen
geboren te Vleuten-De Meern

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CHAPTER 1

General introduction

Many patients in the intensive care unit (ICU) develop generalized weakness, so-called intensive care unit-acquired weakness (ICU-AW).[1] This weakness is caused by dysfunction or damage of muscles (critical illness myopathy), nerves (critical illness neuropathy), or both (critical illness neuromyopathy).[1,2]

The burden of ICU-AW is substantial. About 50% of critically ill patients develop ICU-AW.[2,3] With improving ICU healthcare and ICU survival, it is to be expected that the incidence will rise further in the near future and that many more healthcare providers in- and outside the ICU will encounter patients with ICU-AW.[4] In the past, it was thought that the weakness dissolved when patients recovered from their critical illness, but in recent years it became more and more evident that many patients with ICU-AW do not completely recover and have long-term functional disability for years.[5,6] Furthermore, ICU-AW is associated with longer duration of mechanical ventilation, longer ICU and hospital stay, and increased mortality.[3,5,6] Since no specific treatment to improve or prevent ICU-AW exists, treatment is merely supportive. Early physiotherapy is one of the major supportive measures.[7]

The aims of the research described in this thesis are to investigate the role of inflammation in the pathophysiology of ICU-AW, to develop a new animal model for ICU-AW, and to investigate new methods to diagnose and predict ICU-AW.

INFLAMMATION IN THE PATHOPHYSIOLOGY OF ICU-AW

The pathophysiology of ICU-AW is complex and remains to be elucidated. Several pathophysiological mechanisms are thought to be involved, such as: increased vascular permeability, mitochondrial dysfunction, sodium channel dysfunction, impaired microcirculation, altered intracellular calcium homeostasis, deficient autophagy, and inflammation.[2,8,9]

The main and consistently identified risk factors for ICU-AW are sepsis, systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS), and as such they may share a common pathogenesis with ICU-AW.[3] In these syndromes, an exaggerated and imbalanced systemic inflammatory response plays a central role.[10,11] Therefore, inflammation may also be an important factor in the development of ICU-AW.

Studies investigating inflammation in ICU-AW are bundled in **part I** of this thesis. In **chapter 2** we reviewed the role of systemic inflammation and local inflammation of nerve and muscle tissue in the pathogenesis of ICU-AW. In **chapter 3**, we systematically summarized the current knowledge on inflammation in muscle and nerve tissue in animal models of ICU-AW and in critically ill patients with ICU-AW. In **chapter 4**, we investigated whether patients who develop ICU-AW have a different pattern of systemic inflammation during the first days after ICU admission compared to patients without ICU-AW. As activation of the complement system plays an

important role in the pathogenesis of sepsis, SIRS, MODS and several polyneuropathies, we hypothesized that muscle and nerve damage in ICU-AW may be complement-mediated as well. Therefore, in **chapter 5**, differences in systemic complement activation products between patients with and without ICU-AW were investigated**.**

ANIMAL MODELS FOR ICU-AW

Animal models are essential to further unravel the pathophysiology of ICU-AW and to investigate future treatments. However, existing animal models have serious limitations. Some models are far from the human situation, others have low consistency and reproducibility or are very expensive and time-consuming. In all these models, *in vivo* strength measurements are lacking. This questions the translatability to ICU-AW in humans, as clinical muscle weakness is the hallmark of ICU-AW. We aimed to develop a new, easily applicable mouse model that could serve as an ICU-AW model using *in vivo* strength measurements, further described in **part II** of this thesis. We investigated an *E.coli* sepsis model with young and old mice in **chapter 6** and an *S. pneumoniae* sepsis model in **chapter 7**.

EARLY DIAGNOSIS AND INDIVIDUAL PREDICTION OF ICU-AW

ICU-AW is currently diagnosed clinically with use of the Medical Research Council (MRC) sum score. The MRC score ranges from 0 (no visible contraction) to 5 (normal strength). ICU-AW is defined by a MRC sum score (twelve muscles, three of the upper and three of the lower limbs on both sides) of <48 out of 60, or an average MRC score <4.[1,12] Although this subjective and ordinal score has limitations, it is still considered the best test to diagnose ICU-AW by experts.[12,13] For reliable strength measurements patients need to be awake and attentive.[14] However, patients on the ICU are often sedated and attentiveness is often impaired by for example delirium. This delays the diagnosis of ICU-AW.

An early diagnosis and early prediction of ICU-AW is desirable to allow early prognostication and to enable timely initiation of supportive interventions, like intensive physiotherapy and tracheostomy. Furthermore, in case a specific treatment will become available in the future, an early start of treatment is preferable to prevent structural muscle and nerve damage, since muscle and nerve dysfunction in the early stages may still be entirely reversible.[15] Therefore, other diagnostic methods are needed. Ideally, such a method is easy to perform and easy to repeat on the ICU without being a burden for the patient involved.

In **part III**, studies investigating early diagnosis and early prediction of ICU-AW are described. In **chapter 8** we investigated if ultrasound of muscles and nerves may be used to early diagnose ICU-AW. Muscle and nerve ultrasound is a

noninvasive investigation, which can be easily performed at the bedside on the ICU. In **chapter 9** we externally validated a previously developed prediction model for ICU-AW including three easily and early available clinical predictors.

In **chapter 10** we discuss the main results and implications of this thesis.

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PART I

INFLAMMATION IN INTENSIVE CARE UNIT-ACQUIRED WEAKNESS

CHAPTER 2

The role of local and systemic inflammation in the pathogenesis of intensive care unit-acquired weakness

Esther Witteveen, Marcus J. Schultz, Janneke Horn

INTRODUCTION

A previously healthy 47-year old woman was admitted to the intensive care unit (ICU) with severe pneumosepsis. She received antimicrobial therapy and supportive care consisting of fluid resuscitation, inotropes and vasopressors, and lungprotective ventilation. She developed kidney failure for which continuous venovenous hemofiltration was initiated. She recovered within several days, but after cessation of sedatives it became apparent that she was very weak and could hardly move her limbs. She was clinically diagnosed with intensive care unit-acquired weakness (ICU-AW). After a period of intense rehabilitation she was discharged home, but a year later she still complained of severe impairments in physical functioning. She wondered how a pulmonary infection could have led to extreme weakness of the limbs. You realize that the pathophysiology of ICU-AW is far from understood, but there are suggestions that ICU-AW develops in response to a strong and uncontrolled inflammatory response, as seen with sepsis.

In this chapter, building on a recently published translational review, we aim to provide an overview of studies on local and systemic inflammation in animal models of ICU-AW and in critically ill patients with ICU-AW, and discuss immune-modulating strategies that could benefit patients at risk for or with ICU-AW.[1]

INTENSIVE CARE UNIT-ACQUIRED WEAKNESS

In ICU-AW, a generalized, symmetrical weakness develops, probably already shortly after the onset of a critical illness.[2] It can involve nerves (referred to as critical illness polyneuropathy, CIP), muscles (critical illness myopathy, CIM), or both (critical illness neuromyopathy, CINM).[2] Differentiation between these three entities requires additional invasive investigations, such as electrophysiological investigations or muscle biopsy.[3] This is usually omitted, since so far, it has no clinical consequences: treatment for ICU-AW is merely supportive.

ICU-AW is a very frequent complication of critical illness. A systematic review in 2007, including 24 studies, reported a median incidence of ICU-AW of 57% in patients with sepsis, multiple organ dysfunction syndrome (MODS) or prolonged mechanical ventilation.[4] The incidence of ICU- acquired weakness, though, depends on the patient case mix and the diagnostic criteria used. Incidences up to 100% have been reported in patients with moderate to severe MODS along with sepsis or the systemic inflammatory response syndrome (SIRS).[5] Since survival rates from severe sepsis are improving, it is likely that the incidence of ICU-AW will increase as patients who would have died in the past, now survive and present with ICU-AW.[6,7]

The main and consistently identified risk factors for ICU-AW are SIRS, sepsis, and MODS.[3,4] Multivariable analyses suggested that SIRS and MODS are independently associated with ICU-AW, but this was not confirmed in other studies.[4,8] This finding

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may be because SIRS, sepsis and MODS are highly correlated and some studies only recruited patients with (severe) sepsis. The strong association between MODS and ICU-AW suggests that muscle and nerve dysfunction can be seen as 'another failing organ', and that MODS and ICU-AW have a common cause. An exaggerated and imbalanced systemic inflammatory response is frequently proposed to play a role in the pathogenesis of MODS.[9] Thus, one could hypothesize that an exaggerated and uncontrolled inflammatory response is responsible for the muscle and nerve damage in critically ill patients.

ICU-AW AND SYSTEMIC INFLAMMATION

In SIRS and sepsis, multiple inflammatory pathways are activated, involving inflammatory mediators, complement cascades, acute phase protein release and activation of inflammatory cells and vascular endothelium.[10] While several investigators suggest a role of systemic inflammatory mediators in the pathophysiology of ICU-AW, this has not been studied extensively.

Some studies show at best a possible correlation between elevated plasma cytokine levels (interleukin (IL)-6 and IL-2 receptor) and development of ICU-AW.[11,12] High serum acute phase protein levels and plasma complement products do not correlate with the presence of ICU-AW. [11-14]

The vascular endothelium may be involved in the pathogenesis of ICU-AW, since cytokines, complement membrane attack complex (MAC) and antigen presenting molecules are found on the vascular endothelium in ICU-AW.[1] Along with the presence of adhesion molecules, this indicates endothelial cell activation. Endothelial cell activation and dysfunction are thought to play an important role in the pathogenesis of MODS.[15] Cytokines and other inflammatory mediators induce an increased permeability of vascular endothelium. Due to increased capillary leak, edema forms, impairing tissue oxygenation. Tissue oxygenation is further impaired by microvascular occlusions of neutrophils adhering to the capillary wall by adhesion molecules, causing hypoperfusion and hypoxia. Organ damage in MODS is considered to be a consequence of this indirect damage. These inflammatory mechanisms on the vascular endothelium might also play a role in muscle and nerve tissue in patients with ICU-AW (see figure 1), but this is highly speculative.

While in lung tissue, a massive infiltration of neutrophils causes direct tissue damage by the production of harmful factors, in other organs in MODS, the neutrophils do not enter the tissue.[15] This is in accordance with the finding that in ICU-AW, neutrophils do not infiltrate muscle or nerve tissue, further establishing the idea of muscle and nerve damage in the light of MODS.

It is also suggested that by increased endothelial permeability, a neurotoxic factor in serum might enter the nerves of patients with CIP, but such a factor has not been found.[16]
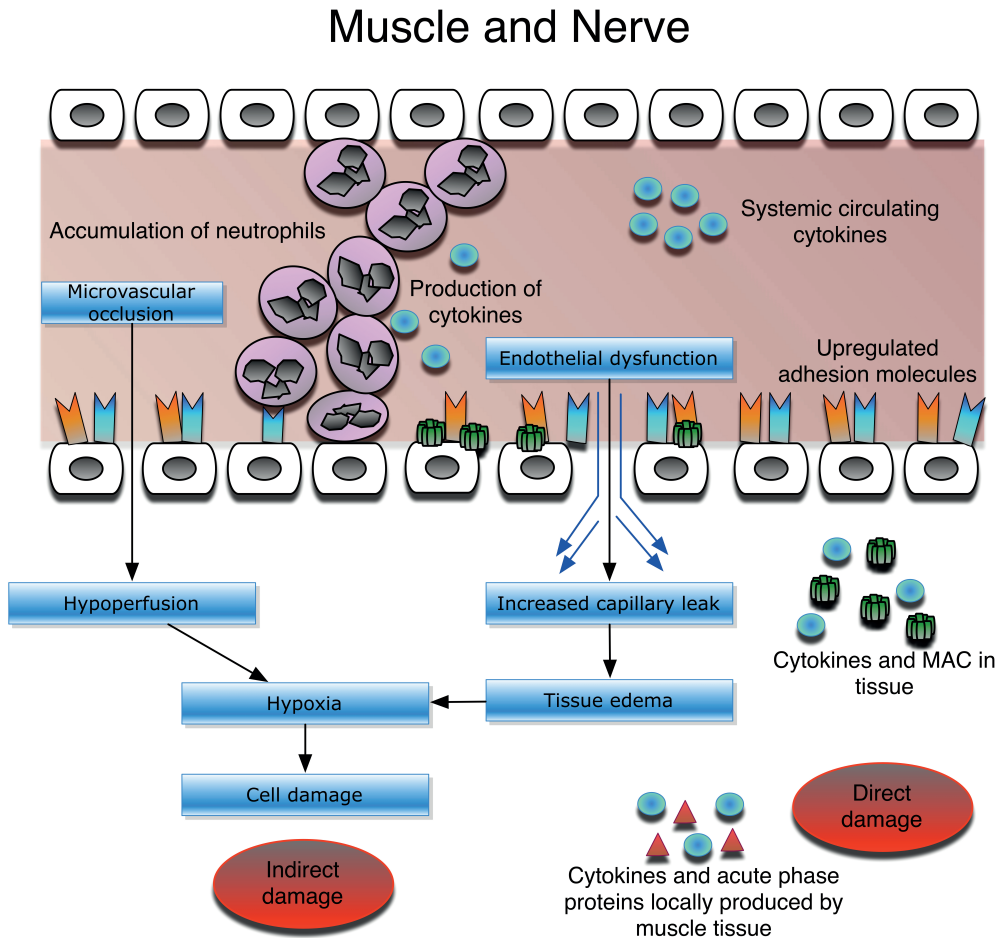
Muscle and Nerve

Figure 1. Inflammatory mediators found in muscle or nerve tissue in ICU-AW and supposed mechanisms of damage (blue boxes).
MAC=membrane attack complex.

ICU-AW AND LOCAL INFLAMMATION IN MUSCLE AND NERVE TISSUE

Muscle inflammation

While cellular infiltrates in muscle tissue have been found in animal models of ICU-AW, these are often absent in muscle biopsies from patients with ICU-AW.[1] The absence of cellular infiltrates differentiates ICU-AW from inflammatory myopathies, like polymyositis and dermatomyositis.[17] Other signs of local inflammation are frequently present, including cytokines, complement factors, antigen presenting molecules and adhesion molecules.[1] The absence of inflammatory cells but presence of these inflammatory mediators suggests that muscle tissue damage may be more a result of locally active inflammatory mediators then of destructive

inflammatory cells. It could be that these inflammatory mediators are produced locally, as skeletal muscle can serve as an exocrine organ expressing and releasing so-called myokines (muscle cytokines) into muscle tissue and blood.[18] In this way, muscle inflammation might even contribute to systemic inflammation.

Notably, elevated cytokine levels in muscle tissue have also been found in ICU patients who do not have ICU-AW.[14] This suggests that the expression of inflammatory mediators in muscle might be common in ICU patients and not specific for ICU-AW.

Nerve Inflammation

There are no animal studies that have investigated local inflammation of nerve tissue in models of ICU-AW, and studies in patients with ICU-AW are scarce. Cellular infiltrates are rarely seen in nerve biopsies of patients with ICU-AW.[1] In one study, high levels of tumor necrosis factor (TNF)-α and adhesion molecules were found in the superficial peroneal nerve of patients with ICU-AW.[19] Nerve biopsies of ICU patients without ICU-AW were not performed.

INFLAMMATORY MEDIATORS IN ICU-AW

Acute Phase Proteins

Elevated plasma C-reactive protein (CRP) levels have been investigated in patients with ICU-AW, but were not associated with ICU-AW.[11-13] Plasma serum amyloid A (SAA) levels were lower in patients with CIM compared to ICU patients without CIM.[14]

Increases in SAA1 and SAA4 expression and SAA1 accumulation in muscle were associated with CIM development. This suggests that SAA is synthesized in the skeletal muscle.[14]

Cytokines

The association between plasma cytokines and development of ICU-AW has been investigated in a few small studies. Increased IL-6 plasma levels (collected 3-7 days and 8-10 days after ICU admission) were an independent risk factor for ICU-AW.[12] This study was hampered by the methodology used. ICU-AW was only defined by abnormal muscle membrane excitability and the sample size was small (22 patients with ICU-AW and 18 without). Moreover, the effect of IL-6 plasma levels was small (hazard ratio of 1.006). Plasma IL-10, insulin-like growth factor (IGF)-1, insulin-like growth factor binding protein-1 (IGFBP)-I and IGFBP-III levels were not independently associated with ICU-AW. In an earlier study, plasma IL-6 and TNF-α levels measured in ICU patients after a diagnosis of ICU-AW were not different from control ICU patients (in whom plasma was measured during the first or second week

of ICU-admission).[20] A correlation between plasma IL-2 receptors and reduction in the amplitude of the compound motor action potential (CMAP) in the median and tibial nerves was found in another study.[11] This correlation was not found for IL-2, IL-6 or IL-10.

Cytokines have also been investigated in muscle and nerve tissue of patients with ICU-AW. In muscle tissue, both the anti-inflammatory cytokine IL-10 and pro-inflammatory cytokines (IL-1β, IL-12, interferon (IFN)-γ and TNF-α-receptor) were found.[21] Cytokines were present on vascular endothelium and in the cytoplasm of muscle fibers of patients with ICU-AW and were absent in two control biopsies of ICU patients without ICU-AW.[21] In nerve tissue from patients with ICU-AW, expression of TNF-α was described in the cytoplasm of endoneurial cell types and in vascular endothelium.[19]

Complement

Plasma C3 and C4 levels did not correlate with CMAP amplitudes of the median, tibial and peroneal nerves.[11] Moreover, complement activation products (C3b/c, C4b/c and C5a) in plasma were not associated with ICU-AW (unpublished data).

In muscle tissue, only MAC (C5b9) has been studied. It was found in a majority of patients with ICU-AW, mainly on vascular endothelium and on necrotic muscle fibers.[21-23] MAC was absent in two control biopsies in ICU patients without ICU-AW.[21]

Adhesion molecules

Positive staining of intercellular adhesion molecules (ICAMs) and vascular cell adhesion molecules (VCAMs) was found in more than 50% of muscle biopsies from patients with ICU-AW.[21] These adhesion molecules were also found in 1 of 2 muscle biopsies of ICU patients without ICU-AW.[21] Therefore, the pathophysiological significance is unknown. E-selectin was not expressed in muscles of patients with ICU-AW or control patients without ICU-AW.[21]

In nerve biopsies of patients with ICU-AW, E-selectin expression was present in 68% (15/22) of biopsies.[19] E-selectin was expressed in the endothelium of epineurial and endoneurial vessels. VCAM-1 and ICAM-1 were present in respectively 100% (22/22) and 77% (17/22) of nerve biopsies. While VCAM-1 and ICAM-1 are expressed under basal conditions, expression of E-selectin in nerves indicates endothelial cell activation.

Antigen presenting molecules

Antigen presenting molecules are frequently found in muscle biopsies of patients with ICU-AW.[21-23] A strong upregulation of both major histocompatibility complex (MHC)-I molecules (human leucocyte antigen (HLA)-1) and MHC-II molecules (HLA-

DR) has been found.[21-23] HLA-DR was expressed on muscle fibers, vascular endothelium and on macrophages in infiltrates. Positive staining for HLA-1 was also seen, as a secondary phenomenon, on atrophic and necrotic muscle fibers.[21,23] HLA-1 was also present in two muscle biopsies from ICU patients without ICU-AW.[21]

IMMUNOMODULATORY THERAPIES AGAINST ICU-AW

So far, only intravenous immunoglobulin (IVIG) has been studied as an immunomodulatory therapy to prevent or mitigate ICU-AW, in one preclinical study and three clinical trials.

In a rat model, IgM-enriched IVIG was given within the first hour after induction of septic peritonitis.[24] Nerve conduction velocity decreased significantly in the untreated group, whereas it did not change in the sham surgery and IVIG group. However, decreased nerve conduction velocity is not typical for ICU-AW.

In a prospective randomized controlled trial, patients with MODS, SIRS or sepsis, and early clinical signs of ICU-AW were randomized to receive IgM-enriched IVIG (0.25 g/kg/day for 3 consecutive days) or placebo (human albumin).[25] The primary outcome in this study was a severity score, consisting of a combination of results of electrophysiological studies and muscle biopsy (and not muscle strength), on day 0 and day 14 after start of treatment. The severity scores deteriorated in both the IVIG and placebo groups. Since no differences in severity scores were found during the interim analysis, this trial was terminated prematurely, after enrolment of 38 patients. The secondary outcomes (28-day mortality and length of ICU stay) were also similar in the two groups.

In a pilot-study in which three patients with an established clinical and electrophysiological diagnosis of ICU-AW were treated with IVIG (0.4 g/kg/day for 5-10 days), patients did not show an improvement in mobilization shortly after immunoglobulin administration.[26] No control patients were included.

Finally, IgM-enriched IVIG was investigated in a prospective cohort study of patients who survived MODS following multiple trauma and, thereafter, developed sepsis due to a nosocomial infection.[27] In eight patients, IVIG (0.3 g/kg/day for 3 days) was given within 24 h after the diagnosis of sepsis. Retrospective chart analysis showed that none of these patients developed ICU-AW (diagnosed by electrophysiological examination at the time of ICU discharge). Eight other patients did not receive IVIG because they were transferred from another hospital or had a delayed diagnosis of sepsis. These patients were used as control patients and ICU-AW was diagnosed in seven of them. Based on these results, the authors suggested that IVIG administration may have prevented the development of ICU-AW.

The effects of blocking various immune mediators on the development of ICU-acquired weakness should be further investigated, either by using specific blocking agents or knock-out animals. The advances in anti-inflammatory therapies are

promising, since more selective immunomodulatory drugs, which target a specific component of the immune system, have been developed.[28]

CHALLENGES AND FUTURE PERSPECTIVES

Little is known about inflammation in muscle or nerve tissue of ICU patients without ICU-AW, because most studies concerning inflammation in ICU- acquired weakness are case series or cohort studies without biopsies of control patients. Therefore, the findings described in this overview might not be specific for ICU-AW but may be seen in all ICU patients. Further research is needed to investigate whether the inflammatory response differs in patients with and patients without ICU-AW.

Furthermore, comparisons between study results are hampered by the different inclusion criteria used and different diagnostic criteria for ICU-AW. Future research should use the current diagnostic criteria for ICU-AW, in which the diagnosis of ICU-AW is primarily based on measurement of manual muscle strength and not on electrophysiological parameters.[2]

As ICU-AW may be as important as other organ failures, such as kidney failure, it deserves far more attention on the ICU. Investigation and documentation of weakness on the ICU according to the present recommendations should be part of standard care.[2,29] In the search for a better understanding of the pathophysiology of ICU-AW and possible therapies, increased awareness of ICU-AW is crucial.

CONCLUSION

Although the established association of ICU-AW with sepsis, SIRS and MODS suggests a role for inflammation, this has not been extensively studied and results so far are inconclusive. Inflammatory mediators have been found in plasma, and muscle and nerve tissue of patients with ICU-AW, and the vascular endothelium seems to be involved. Muscle and nerve injury in ICU-AW may be caused by indirect damage (caused by endothelial dysfunction, as in MODS) and by direct damage by locally produced inflammatory mediators, but this is still speculative. Dysfunction and damage of muscles and nerves in ICU-AW can be seen as failing organs in the spectrum of MODS.

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CHAPTER 3

Muscle and nerve inflammation in intensive care unit-acquired weakness: a systematic translational review

Esther Witteveen, Luuk Wieske, Camiel Verhamme,

Marcus J. Schultz, Ivo N. van Schaik, Janneke Horn

ABSTRACT

Background

Intensive care unit-acquired weakness (ICU-AW) is an important complication of critical illness. The main risk factors, sepsis and the systemic inflammatory response syndrome, suggest an inflammatory pathogenesis. In this systematic translational review we summarize current knowledge on inflammation in muscle and nerve tissue in animal models of ICU-AW and in critically ill patients with ICU-AW.

Methods

We conducted a systematic search in the databases of MEDLINE, EMBASE and Web of Science using predefined search and selection criteria. From the included studies we extracted data on study characteristics and on inflammation in muscle and nerve tissue.

Results

The literature search yielded 349 unique articles, of which twelve animal studies and 20 human studies fulfilled the in- and exclusion criteria. All studies had important shortcomings in methodological quality. In the animal studies, inflammation of muscle tissue was found, represented by cellular infiltration and increased local levels of various inflammatory mediators. In human studies, high levels of various inflammatory mediators were found in muscle and nerve tissue of ICU-AW patients.

Conclusion

This systematic translational review suggests a role for local inflammation in ICU-AW, but the available evidence is limited and studies have severe methodological limitations.

INTRODUCTION

Intensive care unit-acquired weakness (ICU-AW) is an important complication of critical illness and is strongly associated with increased short- and long-term morbidity and mortality.[1] ICU-AW involves nerves (referred to as critical illness polyneuropathy, CIP) or muscles (critical illness myopathy, CIM), but often involves both nerves and muscles (critical illness neuromyopathy, CINM).[2] There is no specific treatment for ICU-AW. Current treatment is merely supportive by treatment of underlying risk factors and intensive physical therapy programs on the ICU.[1,3]

Although ICU-AW has been studied in several animal models and human studies, its pathogenesis still remains poorly understood. Since sepsis and the systemic inflammatory response syndrome (SIRS) are the main and consistently identified risk factors, an inflammatory pathogenesis can be suspected.[4] It is hypothesized that the overwhelming systemic inflammation as seen in sepsis and SIRS causes inflammation in muscles and nerves, resulting in muscle and nerve damage, and ultimately in ICU-AW.

The aim of this systematic and translational review is to investigate whether ICU-AW is characterized by local inflammation in muscle or nerve tissue, or both, in studies of animal models of ICU-AW and studies of critically ill patients with ICU-AW.

METHODS

Search strategy

We searched the MEDLINE, EMBASE and Web of Science databases for animal studies and human studies, using predefined search terms for ICU-AW (and previously used synonyms) combined with terms concerning inflammation (see data supplement, which shows the search terms and restrictions used in the searches). We did not include a specific time period in the search syntax. In addition to the database searches, a search of bibliographies and texts was conducted to identify additional studies. We did not contact authors to provide additional data.

Study selection

Titles and abstracts obtained from the literature search were reviewed to identify potentially relevant studies. By reading the full texts, we selected studies which met the in- and exclusion criteria and we determined their methodological quality. First, an article had to report on an animal model, simulating a critical illness-like condition and testing muscle or nerve functionality (e.g. by electrophysiological investigations or other quantitative measurements for muscle/nerve function), or a series of patients with the clinical diagnosis of ICU-AW (or a previously used synonym). Second, an article had to present data on inflammatory responses in muscle or

nerve tissue, obtained by muscle/nerve biopsy or post-mortem. This didn't have to be the primary outcome of the study.

Non-English studies were included if an English abstract was available. Articles that reported only on *in vitro* investigations, human studies without numeric data pertaining to patients with inflammation, case reports or studies in non-adult patients were excluded from review.

Study characteristics and data extraction

We collected the following information: presence of inflammation, including the presence of inflammatory cells and mediators in muscle or nerve tissue (the primary outcome of this review), types of muscles or nerves investigated, timing of tissue collection, aim of the study and whether nerve or muscle inflammation was the primary outcome of the study.

Additionally, we extracted the following information from articles reporting on an animal study: type of animals used, number of ICU-AW animals and controls used, experimental procedures used, and muscle/nerve functionality tests used. From articles reporting on animal studies testing interventions, we ignored the investigational arms and only collected data from the control groups. From articles reporting on a human study we additionally collected the following: study design, study population, diagnostic criteria for ICU-AW used, number of ICU-AW patients and control patients with biopsy.

In order to obtain an estimate of the frequency of ICU-AW patients who have inflammatory mediators in muscle and/or nerve tissue, we pooled the data from studies reporting on this outcome. We calculated 95% confidence intervals with an online calculator according to the modified Walt method.[5]

Disagreement between reviewers (EW and JH) was resolved by consensus. In this review, patients are described as having ICU-AW instead of subcategory terms, such as CIP, CIM or CINM as used in the original articles, because different criteria were applied for these terms.

RESULTS

Search results

The search was conducted in May 2014. The flowchart of literature search and selection is presented in figure 1. This search identified 349 unique articles. After the additional screening of bibliographies and texts, a total of 12 animal studies[6-17] and 20 human studies[18-37] fulfilled the in- and exclusion criteria.

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┌─────────────────┐  ┌─────────────────┐  ┌─────────────────┐
│ MEDLINE search  │  │ EMBASE search   │  │ WoS search      │
│ 185 articles    │  │ 180 articles    │  │ 170 articles    │
│ identified      │  │ identified      │  │ identified      │
└─────────────────┘  └─────────────────┘  └─────────────────┘
```

Total 535 articles identified by database searches

349 articles after removing duplicates

Animal studies / **Human studies**

31 articles identified / 318 articles identified

29 articles excluded:
  23 no muscle/nerve data on inflammation
  6 no ICUAW/critical illness model

310 articles excluded:
  120 no original data/review
  89 no ICU-AW patients
  64 no muscle/nerve data on inflammation
  25 case report
  9 non-adult patients
  3 no English abstract

2 articles included / 8 articles included

10 articles identified by screening of bibliographies and texts

12 articles identified by screening of bibliographies and texts

12 articles included / 20 articles included

Figure 1. Flowchart study search and selection.
WoS=web of science; ICU-AW=intensive care unit-acquired weakness.

Study characteristics of animal studies

Study characteristics of the 12 animal studies are presented in table 1.[6-17] Different sepsis models were used to induce ICU-AW in various species, including mice[11,15], rats[6-10,12,13,16] and pigs[14,17]. Muscle function was measured by contractile properties[6-8,10,11,15-17] or electrophysiological studies[9,12-14]. *In vivo* quantitative strength measurements were not performed in any of the studies. Inflammation was the primary outcome in two of these studies.[7,15] None of the studies assessed inflammation in nerve tissue.

Study characteristics of human studies

Table 2 presents study characteristics of the 20 human studies included.[18-37] These consisted of 11 case series without a control group[18-26,30,36], six prospective cohort studies[27,29,31,32,35,37] and three case-control studies[28,33,34]. Inflammation was the

primary outcome in four human studies.[28,29,33,37] Study populations ranged from two to 32 patients with ICU-AW. Clinical and electrophysiological criteria used to diagnose ICU-AW were very heterogeneous. Thirteen studies reported on inflammation in muscle tissue[20-23,26-29,31,32,34,35,37], four on inflammation in nerve tissue[18,19,30,33] and three on both[24,25,36]. In all 20 studies combined, 154 muscle biopsies and 69 nerve biopsies were obtained. In eighth studies, a biopsy was taken in a selection of included ICU-AW patients.[18,24,27,29,31,32,35,36]

Twenty-four muscle biopsies[28,29,37] and 20 nerve biopsies[33] were from control patients. Of these control patients 14 muscle biopsies were from ICU-patients without ICU-AW.[29,37] The other control biopsies were from non-ICU patients with neuromuscular diseases (NMD) (n=10 muscle biopsies[28], n=20 nerve biopsies[33]).

Inflammation in muscle tissue in animal studies

In six out of nine studies that reported on the presence of inflammatory cells, cellular infiltrations were found in muscle tissue (table 1).[8-10,12,13,16] One study reported slight inflammatory cell infiltration[10], while the others reported clusters of inflammatory cells[8,12] or moderate neutrophil or inflammatory cell infiltration (4-10 neutrophils per high-power field[13] or 25-42 inflammatory cells per visual field[16]) .

In one of the six studies, inflammatory cells were found in the gastrocnemius and soleus muscles[16], in the other studies, inflammatory cells were found in the diaphragm, mainly in the perivascular area[8-10,12,13]. In one study, inflammatory cells appeared six hours after LPS and disappeared 24 hours after LPS administration.[8] Two studies showed increased cytokine expression in diaphragm.[7,15] In a pig model of sepsis, a more than two-fold upregulation of genes coding for complement factor 3, 4A, 7 and factor B was found, compared to pigs without sepsis.[17] The IL-6 precursor gene and seven chemokine genes were upregulated more than four-fold.

Inflammation in muscle and nerve tissue in human studies

In humans, the presence of inflammatory cells, cytokines and cell adhesion molecules was investigated in both muscle[20-29,31,34-37] and nerve tissue[18,19,24,25,30,33,36], whereas antigen presenting molecules and complement factors were solely investigated in muscle tissue[28,32] (table 2). Data on inflammation (comprising inflammatory cells, antigen presenting molecules, complement factors, cytokines and adhesion molecules) in muscle and nerve tissue of all available ICU-AW patients were pooled (table 3). One study could not be used for pooling, since the number of patients with the inflammatory mediator was not mentioned.[37]

In 18 muscle biopsies of patients with ICU-AW no differences in cytokine expression were found compared to 12 control muscle biopsies of ICU patients without ICU-AW.[37] However, ICU patients with or without ICU-AW had a significantly higher cytokine expression than healthy controls. In another study, two control

muscle biopsies of ICU patients without ICU-AW showed antigen presenting molecules, of which one also showed adhesion molecules.[29] Inflammatory cells, MAC and cytokines were absent in these control biopsies. Muscle biopsies of 10 control NMD patients showed presence of antigen presenting molecules, MAC deposition and inflammatory cells[28] and in another study 20 nerve biopsies of control NMD patients showed expression of adhesion molecules and a cytokine[33]

DISCUSSION

Key findings

Despite the fact that sepsis and SIRS are main risk factors for ICU-AW, this review shows that a detrimental role for local inflammation in muscle or nerve tissue in ICU-AW cannot be established with the available evidence. Although we found many animal and human studies that addressed this topic, these studies have many methodological limitations and direct histological evidence of muscle and nerve inflammation is limited.

The available literature suggests that local inflammation is found in ICU-AW. However, whether this is specific for ICU-AW, cannot be concluded, because appropriate control samples are lacking. Therefore, based on the currently available studies, the exact role of inflammation in ICU-AW remains uncertain.

Inflammatory cell activation and migration in ICU-AW

Clusters of inflammatory cells or moderate inflammatory cell infiltration were reported in muscles in animal models of ICUAW. However, infiltrations of inflammatory cells were absent in most patient muscle biopsies. If present, only infrequent small collections of inflammatory cells were described. Hence, in contrast to inflammatory myopathies like polymyositis and dermatomyositis, cellular infiltration in muscle may not be a characteristic histopathological finding in ICU-AW.[38] Likewise, inflammatory cells were rarely seen in nerve biopsies of ICU-AW patients.

As inflammatory infiltrates are rarely seen in muscle and nerve tissue in patients with ICU-AW, muscle and nerve damage may not be caused directly by inflammatory cell activation, but may result from other sequelae of the inflammatory response, such as inflammatory mediators.

Inflammatory mediators in ICU-AW

In animal muscle tissue, increased cytokine expression and upregulation of complement, chemokine and cytokine genes was found. Inflammatory mediators were also found in muscle and nerve tissue of ICU-AW patients. These comprised cytokines, antigen presenting molecules, MAC and adhesion molecules.

Cytokines mediate muscle damage and play an important role in degeneration and regeneration of the peripheral nervous system after injury.[39,40] In vitro studies also show that beside immune cells, skeletal muscle tissue can release various cytokines contributing to local inflammation.[37,41] As cytokines were also found in muscle biopsies of ICU patients without ICU-AW, but not in healthy patients, cytokine expression might be present in ICU patients irrespective of the presence of ICU-AW.

Inflammatory mediators have been identified on the vascular endothelium. Therefore, endothelial cell activation seems to occur in ICU-AW. Endothelial cell activation can cause endothelial dysfunction and subsequent increased vascular permeability, leading to endoneurial edema causing a disrupted energy and oxygen supply to neurons and ultimately to cell death.[42] This was hypothesized before as a leading cause of CIP.[42] Endothelial dysfunction plays an important role in the pathogenesis of multiple organ dysfunction syndrome (MODS).[43] ICU-AW may be considered as the dysfunction of the neuromuscular system in patients with MODS, supporting that endothelial activation may be involved in its pathogenesis.[1,19]

Strengths and limitations of this systematic review

This is the first systematic review on the inflammatory response at tissue level in ICU-AW, giving a thorough overview of the current knowledge. By including both animal and human studies, we were able to use all available data. The table with pooled results from human studies (table 3) gives an estimate of the frequencies of inflammatory findings in muscle and nerve. These results should be interpreted with caution, since study populations are not completely comparable due to different diagnostic criteria for ICU-AW.

This review has some limitations. The search strategy in MEDLINE, EMBASE and Web of Science did not identify all relevant articles; most of the included articles were identified by search of bibliographies and texts. The inconsistent nomenclature of ICU-AW in previous years, in which more than 18 different terms were used to describe the clinical syndrome of ICU-AW, has formed a major obstacle. Therefore, we included multiple terms for ICU-AW in our search.

Quality of included articles was assessed by presenting study characteristics. A formal quality assessment using a quality assessment tool was not performed, since there is no optimal tool for quality assessment of observational studies including case series.[44] All articles had major inadequacies concerning quality, therefore we decided not to exclude articles based on quality criteria.

Shortcomings of included studies

Besides the lack of control samples, several other issues were identified. Animal studies used different sepsis models, often representing short and acute sepsis,

instead of the more chronic sepsis as seen in patients with ICU-AW. Furthermore, *in vivo* strength measurements were not performed in any of the animal models, while decreased muscle strength is a key feature of ICU-AW. Also, most animal studies investigated the diaphragm and not limb muscles. None of the animal studies investigated inflammation in nerve tissue.

Human studies included very heterogeneous patient populations with different inclusion criteria and different diagnostic criteria for ICU-AW (or CIP, CIM, CINM). We also found a substantial risk for selection bias, since muscle or nerve biopsies were often taken in a subset of included patients. ICU-admission-to-biopsy time differed significantly between studies, and was often not reported at all.

Implications for future research

A better understanding of the pathophysiology of ICU-AW and the role of inflammation is needed to find new potential targets for therapy. To increase knowledge on this topic a clinically relevant and broadly applicable animal model is needed. In this model, *in vivo* weakness has to develop, mimicking the clinical condition of patients with ICU-AW. Such a model would allow further unravelling of the role of different parts of the inflammatory system. Human studies on the role of inflammation in ICU-AW should include a substantial number of patients with ICU-AW and ICU patients without ICU-AW as controls. ICU-AW should be diagnosed using the current diagnostic criteria, completed with adequate electrophysiological tests. Sufficient numbers of muscle and nerve biopsies are needed. Such a study is however challenging, since collection of muscle and nerve biopsies is hampered by coagulopathy, which is often seen in critically ill patients. Nerve biopsies are also controversial because they can induce persistent sensory deficits. Post-mortem nerve and muscle biopsies and new techniques to investigate nerves, like skin biopsies, might be useful in the research of ICU-AW.[45,46]

CONCLUSION

The available evidence cannot establish a detrimental role for local inflammation in muscle or nerve tissue in ICU-AW, because direct histological evidence is limited and studies have several methodological limitations. However, the presence of various inflammatory mediators in muscle and nerve tissue in ICU-AW suggests a role for local inflammation.

CONFLICT OF INTEREST

Prof. I.N. van Schaik received departmental honoraria for serving on scientific advisory boards and a steering committee for CSL-Behring. The other authors declare that they have no competing interests.

Table 1. Study characteristics and inflammatory results of animal studies

Study	Animal	Aim of the study	ICU-AW group	Characterization ICU-AW model	ICU-AW animals n/ with biopsy, n	Control group	Control animals, n/ with biopsy, n	Nerve/ muscle tissue, Type	Time tissue collection	Data inflammatory cells	Data other immune-mediators
Ruff 1984	Rats	Evaluate skeletal muscle protease activity and ability of indomethacin and leupeptin to reduce sepsis-induced muscle wasting	SC injection S. pneumoniae	EDL and soleus contractile properties	13/13	SC injection of heat-inactivated S. pneumoniae	14/14	Muscle, Extensor digitorum and soleus	Day 3 after inoculation	No inflammation in S. pneumoniae rats and control rats	NA
Shindoh 1995	Rats	Examine expression of TNF-α on diaphragm and its relationship with contractile properties	IV injection E. coli endotoxin	Diaphragm contractile properties	48/6	IV saline	8/8	Muscle, Diaphragm	0.5, 1, 1.5, 2, 4 and 6 h after LPS	NA	Increased TNF-α mRNA, to peak at 1.5 h after LPS and return to baseline at 6 h. TNF-α expression in some muscle fibers at 4 and 6 h
Boczkowski 1996	Rats	Examine iNOS induction in the diaphragm and its involvement in diaphragmatic contractile dysfunction	IP injection E.coli LPS	Diaphragm contractile properties	61/61	IP injection saline	20/20	Muscle, Diaphragm	LPS group: 2, 6, 12, 24 and 42 h after LPS Control group: 24 h after saline	Inflammatory cell infiltrations at 6 h after LPS. At 12 h very few and at 24 h no clusters of inflammatory cells. Control group: NR	NA
Lin 1998	Rats	Evaluate nitric oxide in sepsis-mediated diaphragm injury and abnormal myofiber membrane electrophysiology	Aortic injection LPS or CLP	Diaphragm membrane electrophysiology	10/10	Aortic injection saline or sham operation: laparotomy without ligation and puncture	5/5	Muscle, Diaphragm	4 h (LPS) or 24 h (CLP)	Macrophage infiltration, primarily in perivascular areas Largely absent macrophage staining in controls.	NA
Fujimura 2000	Rats	Assess alterations in diaphragmatic contractility and evaluate effect of polyethylene glycol-absorbed superoxide dismutase on the alterations	CLP	Diaphragm contractile properties	6/6	Sham operation: laparotomy without ligation or puncture	6/6	Muscle, Diaphragm	16 h after CLP/sham procedure	Slight inflammatory cell infiltration after CLP. Control group: NR	NA
Divangahi 2004	Mice	Determine function of diaphragm and hind limb muscles and examine its relationship with pulmonary mechanics, pulmonary bacterial burden and level of lung inflammation	IT inoculation P. aeruginosa (high or low dose)	Diaphragm, EDL and soleus contractile properties	12/12	IT inoculation sterile suspension	6/6	Muscle, Diaphragm	Day 2 and 7 after P. aeruginosa	No inflammatory cell reaction and no increase in neutrophils	NA
Donk 2005	Rats	Verify whether glutamine pretreatment protects diaphragm muscle function	CLP	Diaphragm electrophysiological studies	24/24	Sham operation: laparotomy without ligation or puncture	18/18	Muscle, Diaphragm	24, 48 and 72 h after CLP/sham procedure	At 24 h after CLP no neutrophil infiltration, at 48 h and 72 h moderate neutrophil infiltration (4-10 neutrophils per HWP). No neutrophil infiltration at all time points in controls.	NA
Nayci 2005	Rats		CLP	Diaphragm electrophysiological studies	12/12	Sham operation: laparotomy without ligation or puncture	10/10	Muscle, Diaphragm	7 days after CLP/sham procedure	Accumulation of neutrophils and macrophages after CLP. Clusters of inflammatory cells adhering to the wall of post-capillary venules and infiltrating the muscle. Normal appearance in controls	NA

Table 1 continued

	Species	Aim	Intervention	Outcome measures	n	Control	n	Tissue	Timing	Inflammation	Cytokine/gene expression
Noman 2006	Pigs	Develop animal model of acute quadriplegic myopathy	IV E.coli endotoxin, MV, sedation, NMBA and repeated corticosteroids	Extremity electrophysiological studies, myosin-actin protein ratio, myosin-actin mRNA	2/2	MV and sedation	1/1	Muscle, Biceps femoris	5 days after start MV	No inflammation	NA
Divangahi 2007	Mice	Assess diaphragmatic pro-inflammatory mediator gene expression, compare its magnitude and pattern between diaphragm and limb muscle and determine whether IL-10 could suppress expression and improve diaphragmatic force production	IT inoculation with P.aeruginosa (high or low dose)	Diaphragm contractile properties	10/10	IT inoculation sterile suspension	5/5	Muscle, Diaphragm, soleus, EDL and tibialis anterior	48 h after infection	NA	In diaphragm, mRNA upregulation of TNF-α, IL-1alpha, IL-1 beta, IL-6, and IL-18 in high dose infection. No upregulation in low dose infection. In soleus, EDL and tibialis anterior no increase in cytokine expression compared to controls
Fink 2008	Rats	Examine effect of inflammation and immobilization alone and in combination on muscle contraction, histology, and acetylcholine receptor regulation	Repeated IV injections of C. parvum on day 0, 4 and 8. Immobilization of one hind limb or sham immobilization	Hind limb contractile properties	20/20	Repeated IV injections of saline on day 0, 4 and 8 Immobilization of one hind limb or sham immobilization	20/20	Muscle, Gastrocnemius and soleus	12 days after start of C.parvum/ saline injections	Increase in inflammatory cells in C.parvum animals compared to saline animals. If combined with immobilization, increase in inflammatory cells 1.7 and 2.2 times higher, than with inflammation or immobilization alone	NA
Aare 2012	Pigs	Investigate how sepsis aggravates ventilator and immobilization-related limb muscle dysfunction	IV injection of E.coli endotoxin, tracheostomy, MV, sedation, IV fluids, antibiotics	Limb muscle contractile properties (previous experiments)	4/4	Tracheostomy and MV, sedation, IV fluids, antibiotics	4/4	Muscle, Biceps femoris	5 days after endotoxin infusion/ control	NA	Upregulation of complement genes, chemokine genes and IL-6 gene in E.coli animals compared to control animals

ICU-AW=intensive care unit-acquired weakness; SC=subcutaneous; EDL=extensor digitorum longus; TNF-α=tumor necrosis factor-alpha; IP=intraperitoneal; IT=intratracheal; IV=intravenous; LPS=lipopolysacharide; CLP=cecal ligation and puncture; IL=interleukin; iNOS=inducible nitric oxide synthase; E.coli=Escherichia coli; S. pneumoniae=Streptococcus pneumonia; P.aeruginosa=Pseudomonas aeruginosa; C. parvum=Corynebacterium parvum; NMBA=neuromuscular blocking agents; MV=mechanical ventilation; HPW=high power field; NR=not reported; NA=not applicable.

3

Table 2. Study characteristics and inflammatory results of human studies

Study	Study design, P/R	Aim of the study	Study population	Criteria for diagnosis of ICU-AW	ICU-AW patients, n/ with biopsy, n	Control patients	Control patients, n/ with biopsy, n	Nerve/ muscle biopsy, Type	Time of tissue collection (median days after admission, range)	Data inflammatory cells	Data other immune-mediators
Bolton 1984	Case series, R	Document clinical, electrophysiological and morphological characteristics	Severe polyneuropathy within 1 month after ICU admission	NR	5/3	NA	0/0	Nerve (postmortem), Peroneal, vagus, phrenic and CST	3 months, (7 weeks-4 months)	Occasional clusters of inflammatory cells in 1 patient. Scattered mononuclear cells, mainly macrophages in 1 patient	NA
Zochodne 1987	Case series, R	Describe clinical, electrophysiological and pathological experience with polyneuropathy	Polyneuropathy diagnosed by electrophysiological or post-mortem studies	Polyneuropathy diagnosed by electrophysiological or post-mortem studies	9/9	NA	0/0	Nerve (postmortem), NR	NR	No inflammation	NA
Wokke 1988	Case series, NR	Results of light and electron microscopy of intramuscular nerves, motor end plates and muscle	Generalized muscle atrophy and weakness	Generalized muscle atrophy and weakness	2/2	NA	0/0	Muscle, External intercostal	NR	No inflammatory cells or phagocytosis	NA
Griffin 1992	Case series, R	NR	Status asthmaticus with severe generalized weakness	Severe generalized weakness	3/3	NA	0/0	Muscle, Biceps	27, (16-29)	No Inflammatory cells	NA
Gutmann 1996	Case series, R	NR	Acute onset of profound weakness and loss of muscle bulk	Profound weakness and loss of muscle bulk	2/2	NA	0/0	Muscle, NR	31, (30-32)	No inflammatory infiltrates. Occasional necrotic myofibers with myophagocytosis.	NA
Hund 1996	Case series, P	Characterize neuromuscular weaning failure, report clinical signs and outcomes in patients with prolonged dependency on ventilator	Failure to wean from MV unexplained by pulmonary complications	NR	7/3	NA	0/0	Muscle and nerve, NR	NR	No inflammatory infiltrates	NA
Latronico 1996	Case series, P	Identify histological abnormalities in peripheral nerves and muscle	Comatose patients who developed paralysis and absent deep-tendon reflexes	Progressive muscle weakness with minimal movements or complete paralysis, reduced or absent deep tendon reflexes	24/24	NA	0/0	Muscle, Peroneus brevis	21, (8-120)	No inflammatory infiltrates	NA
Hanson 1997	Case series, NR	Increase understanding of mechanisms underlying acute myopathy	Acute myopathy	Severe flaccid quadriplegia and difficulty weaning caused by respiratory muscle weakness	4/4 (4 muscle, 2 nerve)	NA	0/0	Muscle, Vastus lateralis or gastrocnemius Nerve, Sural	25, (21-27) Nerve: NR	Muscle: infrequent small lymphocytic infiltrates in 1 patient. Nerve: no inflammatory lesions, no macrophages	NA
Showalter 1997	Case series, NR	Determine involvement of fast or slow myosin, identify fetal myosin expression, examine structural proteins of muscle fiber and catabolic enzymes in muscle	Profound weakness and requiring MV	Profound muscle weakness and requiring mechanical ventilation	5/5	NA	0/0	Muscle, NR	33, (26-65)	Few small collections of mononuclear cells in 1 patient	NA
Campellone 1998	Cohort, P	Identify frequency of myopathy as a cause of generalized weakness in liver transplant patients	After liver transplantation, at least >14 days on ICU or > 7 days MV	Significant weakness (MRC score 3 or <3 in at least 1 muscle group) without an apparent CNS etiology	8/5	Without significant weakness	69/0	Muscle, Vastus lateralis or deltoid	24, (9-42)	Macrophage infiltration of necrotic fibers uncommonly seen in 4 patients, absent in 1	NA

Table 2 continued

Study	Design	Aim	Inclusion criteria	Weakness definition	N	Control/criteria	Sample	N	Time (days)	Histology (inflammation)	Immunohistochemistry
Bazzi 1999	Case-control, NR	Verify whether muscle necrosis in critically ill is due to an inflammatory process	NR	Critical illness myopathy	5/5	Muscular diseases	Muscle, Quadriceps femoris, tibialis anterior or gastrocnemius	10/10	40 (13-120)	CD4 and CD8 cells rarely present in 3 patients, absent in 2 patients. CD19 (B-cells) absent in 5 patients	HLA-1 and MAC positive in 3 patients, negative in 2 patients
De Letter 2000	Cohort, P	Analyze incidence and investigate role of local immune activation in muscle in development of CIPNM	At least 3 days of MV	Severe tetraparesis (MRC sum score <26/30) with wasting of limbs and hypo- or areflexia; electrophysiological signs of axonal polyneuropathy with or without myopathy	32/30	Without clinical or neurophysiological signs of CIPNM	Muscle, Quadriceps femoris	66/2	NR, > 6 (Within 3 days after diagnosis of ICU-AW)	small infiltrates in 8 patients CD4 cells in 60% of biopsies, CD8 cells in 27%, macrophages in 40% and B-cells in 0% In controls no infiltrates, CD4 cells, CD8 cells, macrophages or B-cells.	ICAM-1 in 58% of biopsies, VCAM 53%, E-selectin 0%, MAC 79%, IL1-beta 71%, IL-12 73%, IFN-gamma 40%, TNFalphaR75 90%, IL-10 96%, HLA-1 100%, HLA-DR 100%. In controls: ICAM-1 and VCAM 50% positive, HLA-1 100%, 0% positive for E-selectin, MAC, IL1-beta, IL-12, IFN-gamma, TNFalphaR75, IL-10 and HLA-DR
De Jonghe 2002	Cohort, P	Assess clinical incidence, risk factors, and outcomes of ICU-acquired paresis	MV for at least 7 days, available for muscle strength testing	MRC sum score <48/60 at 7 days after awakening	24/10	MRC sum score >48/60 at 7 days after awakening	Muscle, Quadriceps or deltoid	71/0	NR, > 21	Inflammatory reaction in 1 patient	NA
Sander 2002	Case series, R	NR	Patients with quadriparesis and hyporeflexia	Quadriparesis and hyporeflexia	8/8	NA	Nerve, Sural	0/0	NR	No inflammatory infiltrates	NA
Bednarik 2003	Cohort, P	Evaluate spectrum and time profile of electrophysiological parameters in neuromuscular involvement in critically ill and correlation with biopsy	Failure of at least two organ systems	Electrophysiological signs of new or progressing neuropathy and/or myopathy	26/11	No electrophysiological signs of new or progressing neuropathy and/or myopathy	Muscle, Quadriceps femoris or tibialis anterior	20/0	NR	NA	Anti-HLA-1 and anti-MAC in 8 patients, absent in 3 patients
Fenzi 2003	Case-control, P	Analyze expression of endothelial-cell adhesion molecules and tumor necrosis factor alpha in nerve microvessels.	Comatose patients with clinical signs of NMD	Progressive muscle weakness with minimal movements or complete paralysis, reduced or absent deep tendon reflexes	22/22	NMD (non-ICU-patients)	Nerve, Superficial peroneal nerve	20/20	21, (8-120)	In patients no inflammatory infiltrates, In controls NR.	E-selectin in epineurial vessels in 68% of patients, in endoneurial vessels in 54%, VCAM-1 in epineurial and endoneurial vessels in 77%, ICAM-1 in epineurial vessels in 100%, TNF-α detected in 95%. E-selectin in epineurial vessels in 35% of controls, in endoneurial vessels in 20%, VCAM-1 in epineurial and endoneurial vessels in 70%, ICAM-1 in epineurial vessels in 100%, TNF-α in 100%.
Stibler 2003	Case-control, NR	Develop rapid method to quantify myosin in muscle biopsy specimens	Patients with generalized flaccid muscle weakness with sparing of cranial muscles	Generalized flaccid muscle weakness with sparing of cranial muscles	11/11	muscle biopsy for various symptoms	Muscle, Tibialis anterior or vastus lateralis	47/0	32, (16-104)	No inflammatory cells	NA
Amaya Villar 2005	Cohort, P	Determine incidence, risk factors and impact on outcome of acute quadriplegic myopathy in critically ill	Exacerbation of COPD requiring MV and intravenous corticosteroids	Small, brief, polyphasic MUAPs; representing at least 20% of all MUAPs recorded	9/3	Without electrophysiological criteria for ICU-AW	Muscle, Biceps brachii	17/0	NR (>2-12 days after start of MV)	No inflammatory changes	NA
Marchiori 2006	Case series, P	Describe neurological involvement in SIRS	Patients with SIRS referred to neurologists	NR (clinical diagnosis of NMD)	28/9 (9 muscle, 2 nerve)	NA	Muscle, NR Nerve, Sural	0/0	NR NR	Muscle: no inflammatory changes Nerve: no inflammatory changes	NA

3

Table 2 continued

| Langhans 2014 | Cohort, P | To test the relevance of IL-6 and SAA1 expression in muscle of patients at risk for CIM | Patients with SOFA scores ≥8 on 3 consecutive days within 5 days in ICU | CMAP after direct muscle stimulation <3 mV | 18/18 | CMAP after direct muscle stimulation >3 mV | 12/12 | Muscle, Vastus lateralis | 5 (early time point) and 15 (late time point) | NA | No difference in IL-6 and TNF-α mRNA expression between CIM and non-CIM patients. |

ICU-AW=intensive care unit-acquired weakness; R=retrospective; P=prospective; NR=not reported; NA=not applicable; ICU=intensive care unit; MV=mechanical ventilation; MRC=medical research council; CST=cervical sympathetic trunks; NMD=neuromuscular disease; CNS=central nervous system; CIM=critical illness myopathy; CIPNM=critical illness polyneuromyopathy; COPD=chronic obstructive pulmonary disease; SIRS=systemic inflammatory response syndrome; SOFA=sequential organ failure assessment; HLA=human leukocyte antigen; MAC=membrane attack complex; ICAM=intercellular adhesion molecule; VCAM=vascular cell adhesion molecule; IL=interleukin; TNF-α=tumor necrosis factor-alpha; IFN=interferon; SAA1=serum amyloid A1; MUAP=motor unit action potential; CMAP=compound muscle action potential.

Table 3. Pooled data of inflammation in muscle and nerve tissue in ICU-AW patients.

Data of inflammation in muscle and nerve tissue of all ICU-AW patients of all human studies was summated. We calculated the number of studies in which a mediator was investigated and the overall percentage of ICU-AW patients with the presence of an inflammatory mediator (total number of patients with the mediator divided by the total number of patients with data on the presence of that mediator). Percentages are presented with 95% confidence intervals.

	Muscle				Nerve			
	Number of studies	Total ICU-AW patients	Patients with positive finding, n	Percentage with positive finding (95%CI)	Number of studies	Total ICU-AW patients	Patients with positive finding, n	Percentage with positive finding (95%CI)
Any inflammatory cells	**14**	**116**	**29**	**25 (18-34)**	**7**	**46**	**2**	**4 (0-15)**
CD4 positive cells	2	35	21	60 (44-74)				
CD8 positive cells	2	35	11	31 (18-48)				
B-cells	2	34	0	0 (0-12)				
Macrophages	2	35	16	46 (30-62)				
Any antigen presenting molecules	**3**	**44**	**40**	**91 (79-97)**				
HLA-1	3	44	39	89 (76-96)				
HLA-DR	1	29	29	100 (86-100)				
Any complement factors	**3**	**44**	**32**	**73 (58-84)**				
MAC	3	44	32	73 (58-84)				
Any cytokines	**1**	**30**	**28**	**93 (78-99)**	**1**	**22**	**21**	**95 (76-100)**
IL1-beta	1	28	20	71 (53-85)				
IL-12	1	26	19	73 (54-87)				
IFN-gamma	1	25	10	40 (24-59)				
TNF-alpha receptor	1	30	27	90 (74-97)	1	22	21	95 (76-100)
IL-10	1	28	27	96 (80-100)				
Any adhesion molecules	**1**	**30**	**21**	**70 (52-83)**	**1**	**22**	**22**	**100 (82-100)**
E-selectin	1	26	0	0 (0-15)	1	22	15	68 (47-84)
ICAM-1	1	26	15	58 (39-74)	1	22	22	100 (82-100)
VCAM-1	1	30	16	53 (36-70)	1	22	17	77 (56-90)

ICU-AW=intensive care unit-acquired weakness; CI=confidence interval; HLA=human leukocyte antigen; MAC=membrane attack complex; IL=interleukin; TNF=tumor necrosis factor; IFN=interferon; ICAM=intercellular adhesion molecule; VCAM=vascular cell adhesion molecule.

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DATA SUPPLEMENT

Search terms and restrictions used in the MEDLINE search

(((inflammatory OR inflammation OR infiltrate OR "immune system" OR "immune activation" OR cytokines OR interleukins OR chemokines OR "cell adhesion molecule"OR "adhesion/activation molecules" OR "major histocompatibility complex" OR mhc OR complement OR "membrane attack complex"OR mac OR c5b9)))

AND

((((((((critical[Title/Abstract] AND illness[Title/Abstract]) OR "critical illness"[Title/Abstract]) AND (myopathy[Title/Abstract] OR neuropathy[Title/Abstract] OR polymyopathy[Title/Abstract] OR polyneuromyopathy[Title/Abstract] OR polyneuropathy[Title/Abstract])))) OR ((((intensive[Title/Abstract] AND care[Title/Abstract] AND unit[Title/Abstract]) OR "intensive care unit"[Title/Abstract]) AND (acquired[Title/Abstract] AND (weakness[Title/Abstract] OR paresis[title/abstract]))))))) OR (("acute myopathy"[Title/Abstract] OR "acute quadriplegic myopathy"[Title/Abstract] OR "critical illness myopathy"[Title/Abstract] OR "critical illness neuropathy"[Title/Abstract] OR "critical illness polyneuropathy"[Title/Abstract] OR "critical illness polyneuromyopathy"[Title/Abstract] OR "critical illness neuromyopathy"[Title/Abstract])))

Search terms and restrictions used in the EMBASE search

(Inflammatory or inflammation or infiltrate or 'immune system' or 'immune activation' or cytokines or interleukins or chemokines or 'cell adhesion molecule' or 'adhesion molecules' or 'activation molecules' or 'major histocompatibility complex' or mhc or complement or 'membrane attack complex' or mac or c5b9).af.)

AND

(((((critical and illness) or "critical illness") and (myopathy or neuropathy or polymyopathy or polyneuromyopathy or polyneuropathy)) or (((intensive and care and unit) or "intensive care unit") and (acquired and (weakness or paresis))) or ("acute myopathy" or "acute quadriplegic myopathy" or "critical illness myopathy" or "critical illness neuropathy" or "critical illness polyneuropathy" or "critical illness polyneuromyopathy" or "critical illness neuromyopathy")).ti,ab.)

Search terms and restrictions used in the Web of Science search

TOPIC: ((((inflammatory OR inflammation OR infiltrate OR immune system OR immune activation OR cytokines OR interleukins OR chemokines OR cell adhesion moleculeOR adhesion/activation molecules OR major histocompatibility complex OR mhc OR complement OR membrane attack complexOR mac OR c5b9))))

AND

TOPIC: (((((((((critical AND illness) OR 'critical illness') AND (myopathy OR neuropathy OR polymyopathy OR polyneuromyopathy OR polyneuropathy)))) OR ((((intensive AND care AND unit) OR 'intensive care unit') AND (acquired AND (weakness OR paresis)))))) OR (('acute myopathy' OR 'acute quadriplegic myopathy' OR 'critical illness myopathy' OR 'critical illness neuropathy' OR 'critical illness polyneuropathy' OR 'critical illness polyneuromyopathy' OR 'critical illness neuromyopathy'))))

CHAPTER 4

Increased early systemic inflammation in intensive care unit-acquired weakness; a prospective observational cohort study

Esther Witteveen, Luuk Wieske, Tom van der Poll, Marike van der Schaaf, Ivo N. van Schaik, Marcus J. Schultz, Camiel Verhamme, Janneke Horn, on behalf of the MARS consortium

ABSTRACT

Objective

To investigate whether patients who develop intensive care unit-acquired weakness (ICU-AW) have a different pattern of systemic inflammatory markers compared to critically ill patients who do not develop ICU-AW.

Design

Prospective observational cohort study

Setting

Mixed medical-surgical ICU of a tertiary care hospital in the Netherlands

Patients

Newly admitted critically ill patients, ≥48 h on mechanical ventilation with a non-neurological ICU admission diagnosis were included.

Interventions

A panel of systemic inflammatory markers and soluble vascular adhesion molecules were measured in plasma samples of day 0, 2 and 4 after ICU admission. ICU-AW was diagnosed by manual muscle strength testing as soon as patients were awake and attentive.

Measurements and main results

Ninety-nine of 204 included patients developed ICU-AW. Principal component regression analysis, adjusted for confounders, showed that principal component 1, mainly loaded with IL-6, IL-8, IL-10 and fractalkine, was significantly higher in patients who developed ICU-AW (odds ratio 1.35 (95% confidence interval 1.18-1.55)). Partial least squares-discriminant analysis also showed that these markers were the most important discriminative markers. Mixed-effects models of these markers showed that ICU-AW was associated with an independent 1.5 to 2-fold increase in these markers.

Conclusions

Systemic inflammation is increased in patients who develop ICU-AW compared to patients who do not develop ICU-AW in the first four days after ICU admission. This finding is consistent when adjusted for confounders, like disease severity. A group consisting of IL-6, IL-8, IL-10 and fractalkine, were identified to be the most important.

INTRODUCTION

Intensive care unit-acquired weakness (ICU-AW) is a serious complication of critical illness[1] and causes increased morbidity and mortality.[2,3]

The exact pathogenesis of ICU-AW is unidentified and probably multifactorial. As sepsis, the systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS) are the main risk factors, an inflammatory pathogenesis is assumed.[4]

A key element of these risk factors is activation of systemic inflammatory pathways, such as induction of cytokines.[5] The development of ICU-AW might be associated with the extent to which these factors are activated.

Elevated systemic levels of cytokines and endothelial cell activation can induce increased permeability of vascular endothelium leading to MODS.[6] This may also lead to impaired oxygenation of muscle and nerve tissue, causing muscle and nerve damage.[7] Inflammatory mediators and endothelial cell activation markers have been found in muscle and nerve tissue of patients with ICU-AW, but histological evidence is limited.[8] However, whether an association exists between increased systemic inflammation and development of ICU-AW remains unclear. A limited number of systemic inflammatory markers were investigated previously in patients with ICU-AW.[9] These studies showed varying results and in none of them patterns of inflammatory markers were investigated.

To explore systemic inflammation as a possible pathophysiological mechanism in ICU-AW, we investigated whether critically ill patients who develop ICU-AW have a different pattern of systemic inflammatory markers in the first four days after ICU admission, compared to critically ill patients who do not develop ICU-AW. We did not aim to predict the presence or absence of ICU-AW.

METHODS

Design and ethical approval

This prospective observational study was performed within the framework of the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) study (ClinicalTrials.gov, NCT01905033). The Institutional Review Board gave approval for an opt-out consent method (protocol number 10-056C).

Study setting

The study was performed in a mixed medical-surgical ICU of the Academic Medical Center in Amsterdam. Several standards of care are applied in this ICU, such as glucose control between 90mg/dl and 144mg/dl, restricted use of neuromuscular blocking agents and early mobilization. Sedation is stopped as soon as possible.

In- and exclusion criteria

Patients newly admitted to the ICU between 1 January 2011 and 31 December 2012, ≥18 years old, were eligible for inclusion. We included consecutive patients who were ≥48 hours on mechanical ventilation. Patients admitted because of stroke, traumatic brain or spinal injury, a neuromuscular disorder, central nervous system infection or cardiac arrest were excluded. Patients with pre-existing spinal injury or poor pre-hospital functional status (modified Rankin score[10] >3) were also excluded. For this manuscript, only patients with both blood samples and the outcome (ICU-AW or no ICU-AW) available were analyzed.

Collection of clinical data and blood samples

Data on patient and disease characteristics were prospectively collected by trained observers.[11] Presence of sepsis was scored when patients had SIRS (according to the Bone criteria[12]) and antibiotic administration for the suspicion of an infection. Immune insufficiency at admission was defined by use of immunosuppressive medication at admission, and/or chemo/radiotherapy in the year before ICU admission and/or a documented humoral or cellular immune deficiency.

All patients with sepsis were managed according to protocols following the Surviving Sepsis Campaign guidelines.[13]

Blood samples were collected from leftover plasma drawn for routine care. Plasma samples were stored at -80 °C within 4 hours after collection from the patient.

Muscle strength assessment

Trained physiotherapists performed manual muscle strength testing (MMT), using the Medical Research Council (MRC) scale. As soon as patients were awake (defined as Richmond Sedation and Agitation Scale (RASS) between -1 and 1) and attentive (able to adequately respond to verbal commands with eyelids), six muscle groups were tested bilaterally: shoulder abductors, elbow flexors, wrist flexors, hip flexors, knee extensors and ankle dorsiflexors. Our outcome ICU-AW was defined as a mean MRC score <4, in accordance with the international consensus statement.[2]

Inflammatory marker assays

All inflammatory marker measurements were done in EDTA anticoagulated plasma obtained within 24 hours after ICU admission (day 0) and on day 2 and 4 after ICU admission. We analyzed a panel of inflammatory markers, all of which are assumed to play a role in sepsis. The panel consisted of pro- and anti-inflammatory cytokines, a chemokine and soluble vascular adhesion molecules: interleukin-1 beta (IL-1β), IL-6, IL-8, IL-10, IL-13, tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ),

granulocyte macrophage colony-stimulating factor (GM-CSF), fractalkine, soluble intercellular adhesion molecule-1 (sICAM-1), soluble E-selectin (sE-selectin) and soluble P-selectin (sP-selectin). For further details on these measurements see the data supplement.

Statistical analysis

See the data supplement for details on the statistical methods. We compared two outcomes: ICU-AW or no ICU-AW. Unless otherwise stated, levels of inflammatory markers of all time points together were used for analyses.

As the inflammatory response is a cascade of activated inflammatory markers, we considered it better to look at patterns of markers instead of solitary markers, since these are highly correlated. In this way we looked at the whole complex network and avoided multiple testing. Multiple methods to investigate patterns of inflammatory markers and their association with ICU-AW were used. First, to visualize patterns, a heat map was created with hierarchical clustering of columns (inflammatory markers).

Secondly, principal component regression analyses[14,15] were performed with a priori selected possible confounders for ICU-AW. Based on the literature we used age, gender, presence of sepsis, immune insufficiency at ICU admission, corticosteroid use and Sequential Organ Failure Assessment (SOFA) score. By inclusion of the SOFA score, which is a score for degree of organ failure based on six organ systems, we prevented the use of too many variables in our model. The Acute Physiology and Chronic Health Evaluation IV score (APACHE IV) was not included in the model because of collinearity with the SOFA score. We also visualized the course of principal component (PC) 1-3 over time and assessed the difference between patients with and without ICU-AW with linear mixed-effects models.

Next, partial least squares-discriminant analysis (PLS-DA) was used to get a maximum separation of components between patients with and patients without ICU-AW. We also used PLS-DA for different ICU-AW severities (severe ICU-AW: mean MRC<3, moderate ICU-AW: mean MRC<4 and >3) and an analysis stratified by sepsis.

To quantify the effect of the inflammatory markers and to account for the repeated measurements structure in our data we used mixed-effects models. To restrict multiple testing, only the variables with a Variable Importance in Projection (VIP) >1 in the PLS-DA were selected for mixed-effects models. The effect of ICU-AW on selected inflammatory markers, adjusted for confounders, was assessed.

As a sensitivity analysis, we assessed the influence of missing values.

RESULTS

eFigure 1 in the data supplement shows the flowchart of screened and included patients. For this study, MRC measurements and plasma samples were available for 204 patients, of whom 99 patients developed ICU-AW. Patient characteristics are presented in Table 1.

Table 1. Patient characteristics

	ICU-AW n=99	No ICU-AW n=105	P-value
Male (%)	50 (50.5)	66 (62.9)	0.101
Age (median [IQR])	64.0 [55.5, 72.0]	61.0 [50.0, 70.0]	0.070
Admission type (%)			0.469
medical	57 (57.6)	62 (59.0)	
planned surgical	16 (16.2)	22 (21.0)	
emergency surgical	26 (26.3)	21 (20.0)	
Systemic inflammatory respons syndrome*	99(100)	102(97.1)	0.266
Any sepsis*	87 (87.9)	75 (71.4)	0.006
Primary site of infection			0.010
pulmonary	40 (46.0)	38 (50.7)	
cardiovascular	6 (6.9)	9 (12.0)	
abdominal	29 (33.3)	9 (12.0)	
urinary tract	1 (1.1)	5 (6.7)	
other	11 (12.6)	14 (18.7)	
Immunodeficiency prior to ICU admission	32 (32.3)	29 (27.6)	0.562
Corticosteroids on ICU*	80 (80.8)	60 (57.1)	<0.001
Acute Physiology and Chronic Health Evaluation IV Score (median [IQR])	90.0 [74.5, 103.0]	69.0 [56.0, 95.0]	<0.001
Maximum sequential organ failure assessment score on sample day (mean (sd))	11.7 (3.6)	9.2 (3.4)	<0.001
Average MRC score (median [IQR])	2.5 [1.3, 3.2]	4.7 [4.0, 5.0]	NA
Days from ICU admission to MRC (median [IQR])	9.0 [6.0, 16.0]	7.0 [5.0, 9.0]	<0.001
Length of stay ICU (median [IQR])	16.0 [8.0,27.0]	7.0 [5.0, 11.0]	<0.001
Mechanical ventilation duration (median [IQR])	13.0 [6.0, 22.0]	6.0 [4.0, 8.0]	<0.001
Death in ICU (%)	15 (15.2)	5 (4.8)	0.024

* During first 4 days after ICU-admission
ICU-AW=intensive care unit-acquired weakness; IQR=interquartile range; MRC=medical research council.

After Bonferroni correction, IL-1β, IL-6, IL-8, IL-10, IFNγ, fractalkine and sICAM-1 were significantly higher in patients with ICU-AW (eTable 1 in the data supplement). Graphs of inflammatory marker values over time are presented in eFigure 2 in the data supplement. Several measurements of IL13, GM-CSF, TNFα and IFNγ were below detectable limits (eTable 2 in the data supplement).

Roughly three main clusters of inflammatory markers were identified using heat map analyses (Figure 1): cluster 1 (including IL-6, IL-8, IL-10, IFNγ and fractalkine), cluster 2 (GM-CSF, IL-1β, TNFα, IL-13) and cluster 3 (sP-selectin, sE-selectin, sICAM-1). Especially markers in cluster 1 were higher in patients with ICU-AW (Figure 1, panel B).
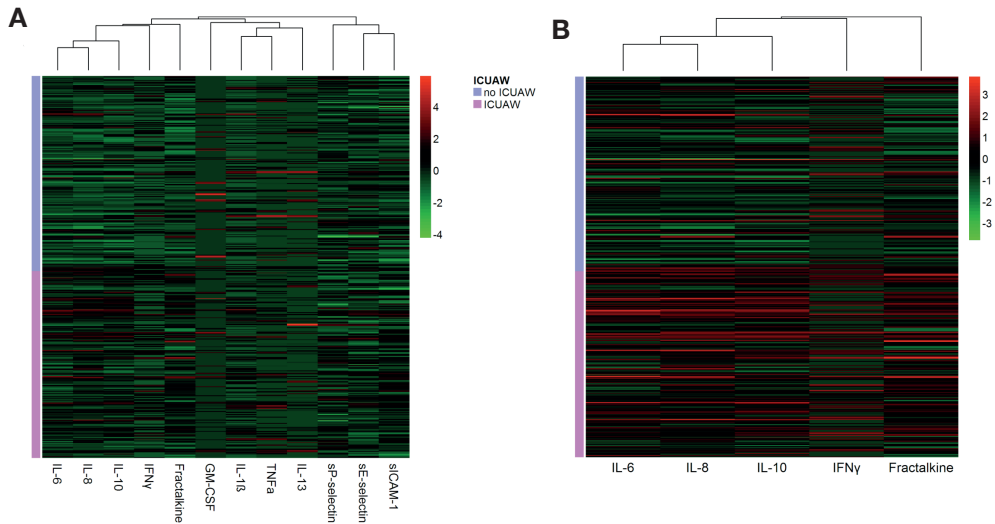

Figure 1. Heat maps of inflammatory markers.
Standardized values of inflammatory markers with hierarchical clustering of columns (A; all markers, B; selected markers). Each row represents a single measurement of a patient. Rows are sorted on presence of ICU-acquired weakness (ICU-AW): rows with *purple* in front are patients without ICU-AW (*n*=275 measurements), rows with *pink* are patients with ICU-AW (*n*=263 measurements). Roughly, three main clusters can be identified in A. The markers in cluster 1 appear higher in patients with ICU-AW (B).
GM-CSF=granulocyte macrophage colony-stimulating factor; IL=interleukin; IFNγ=interferon gamma; sICAM=soluble intercellular adhesion molecule; TNF=tumor necrosis factor.

PC regression analysis showed that the first three PCs accounted for 57.7% of the variance in the data. All PCs showed a significant difference between patients with and without ICU-AW (Table 2). PC1, loaded by IL-6, IL-8, IL10 and fractalkine, had the largest effect size (OR 1.35 (95% CI 1.18-1.55). Loading coefficients are presented in eTable 3 in the data supplement.

Table 2. Principal component regression analysis
Multivariable logistic regression with three principal components, unadjusted and adjusted for confounders

PC	Crude OR (95% CI)	Adjusted OR (95% CI)	Main loadings of PC
PC1	1.46 (1.31-1.63)	1.35 (1.18-1.55)	IL-6, IL-8, IL-10, Fractalkine
PC2	1.18 (1.03-1.36)	1.17 (1.02-1.35)	(Negatively loaded) IL-1, IL-13, TNFa
PC3	0.81 (0.70-0.94)	0.85 (0.72-0.99)	sICAM-1, sP-selectin, sE-selectin

IL=interleukin; OR=odds ratio; PC=principal component.

eFigure 3 in the data supplement shows the course of PC1 to PC3 over time. PC1 is significantly higher in patients with ICU-AW than in those without (P=<0.0001, P adjusted=0.0001).

PLS-DA identified IL-6, IL-8, IL-10 and fractalkine to be the most important discriminative markers (VIP scores: IL-8 1.56, fractalkine 1.45, IL-10 1.40, IL-6 1.20). Scores and loadings plots of the first two components of PLS-DA are presented in Figure 2 (Panel A and B).

Although there is some variation in loadings between the different time points, IL-6, IL-8, IL-10 and fractalkine are consistently identified as the most important markers (Figure 2, panel C). On the first day of ICU-admission, IL-1β and IFNγ have also a VIP>1. This was not found at the other time points.

PLS-DA analysis for groups in which ICU-AW was split in severe and moderate ICU-AW showed that the first component (including IL-6, IL-8, IL-10, Fractalkine, ICAM and IL-13) was increasing with increasing ICU-AW severity (eFigure4).

Separate PLS-DA for patients with sepsis and without sepsis showed similar results, with IL-6, IL-8, IL-10 and fractalkine as the most discriminant markers (eFigure 5).

Mixed-effects models showed that ICU-AW was associated with an independent 1.5 to 2-fold increase in the markers IL-6, IL-8, IL-10 and fractalkine (Table 3).
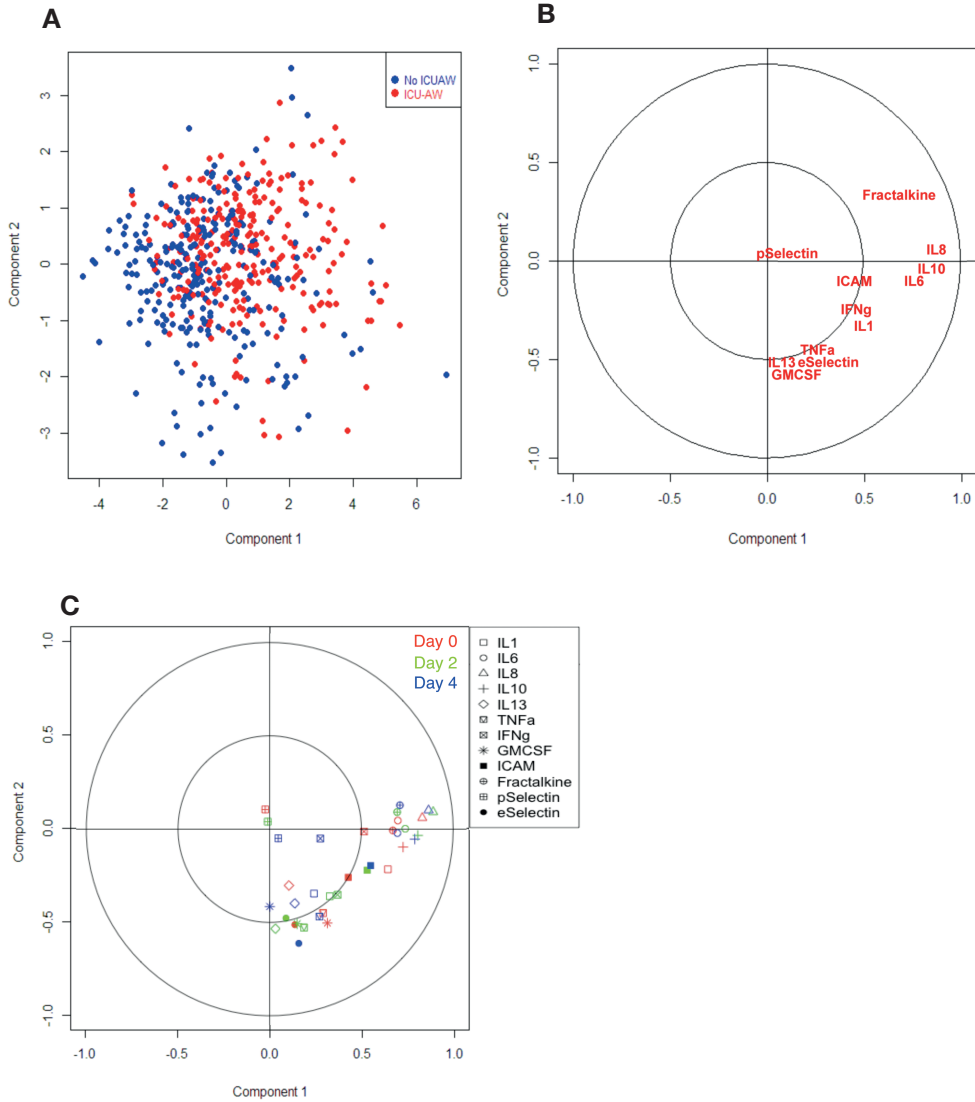
A

B

4

C

Figure 2. Scores plot and loadings plot of PLS-DA
Scores plot (A) and loadings plot (B) of first 2 components of PLS-DA of all plasma samples. Loadings plot of all time points (C). Patients with ICU-AW have a higher score on component 1 (A). IL-8, IL-10, IL-6 and fractalkine are the highest loaded on component 1 (B and C).
GMCSF=granulocyte macrophage colony-stimulating factor; IFNg=interferon gamma; ICAM=intercellular adhesion molecule; TNF=tumor necrosis factor.

Table 3. Mixed-effects models
Linear mixed-effects models showing the association between ICU-AW and inflammatory markers. Predicted effects are fold increase in pg/ml in patients with ICU-AW compared to patients without ICU-AW.

Marker	Predicted effect of ICU-AW (95% CI) (fold-increase)	Pooled effect of ICU-AW (95% CI) imputed data sets (fold-increase)
IL-6	2.15 (1.39-3.32)	2.34 (1.38-3.96)
IL-8	2.27 (1.60-3.21)	2.21 (1.44-3.41)
IL-10	1.86 (1.34-2.57)	1.85 (1.21-2.83)
Fractalkine	1.65 (1.25-2.17)	1.57 (1.11-2.23)

ICU-AW=intensive care unit-acquired weakness; IL=interleukin.

In the principal component regression analysis of imputed datasets only PC1 remained significantly higher between patients with and patients without ICU-AW (OR 1.33 (95% CI 1.17-1.49). In all imputed datasets PC1 was loaded by IL-6, IL-8 and IL10, and in six of ten imputed datasets also by fractalkine. PC2 and PC3 were not different in imputed datasets.

PLS-DA of imputed datasets showed corresponding results with the non-imputed dataset, with IL-6, IL-8, IL-10 and fractalkine having VIP scores>1 in all imputed datasets.

In the mixed-effects models, imputed datasets did not change the estimates for the effect of ICU-AW (Table 3).

DISCUSSION

To our knowledge, this is the first study investigating patterns of systemic inflammatory markers in ICU-AW. The results show that in the first four days after ICU admission systemic inflammation is increased in critically ill patients who develop ICU-AW compared to critically ill patients who do not develop ICU-AW. Despite the fact that there is not a clear decision boundary between patients with and patients without ICU-AW in the biplot, a group of four markers, IL-6, IL-8, IL-10 and fractalkine, were identified to be the most discriminant at all time points, including at ICU admission. As ICU-AW is not yet present at ICU admission, this suggests that these increased markers may cause ICU-AW and are not a consequence of ICU-AW. Besides, inflammatory markers increased with increasing ICU-AW severity.

The presence of ICU-AW was associated with a 1.5 to 2-fold increase in these markers. This increase seems to be independent of potential confounders for development of ICU-AW, indicating that levels of IL-6, IL-8, IL-10 and fractalkine are higher in patients with ICU-AW, irrespective of disease severity.

Comparisons with previous studies

Previous studies investigated a limited number of systemic inflammatory markers and none of them investigated patterns.

Two studies showed an association between plasma cytokines and electrophysiological measurements in ICU patients.[16,17] Unfortunately, a diagnosis of ICU-AW based on MMT, which is the preferred method according to expert based guidelines[18,19], was not made in these studies. One study investigated IL-6 and IL-10 in 22 ICU patients with abnormal membrane excitability as a marker of myopathy and 18 patients with normal excitability.[16] IL-6 was found to be an independent risk factor, with a small hazard ratio of 1.006 (95% CI 1.003-1.009). For IL-10 no difference was found. In another study, in 20 ICU patients, IL-2 receptor levels were negatively correlated with compound muscle action potential amplitudes of median and tibial nerves.[17] This was not found for IL-2, IL-6, IL-10 and complement factors C3 and C4.

Some studies did not find any differences in plasma cytokines. No associations were found between MMT composite scores of 36 ICU patients and sequentially measured cytokines IL-8, IL-15 and TNFα on three consecutive days from day 6 after ICU admission.[20] In another study mean and maximum levels of IL-6 and TNF did not differ between nine ICU patients with critical illness polyneuropathy (CIP) and ten ICU patients without CIP.[21] However, blood samples were taken after a longer duration of critical illness in patients with CIP (ranging from 12 to 55 days) compared to patients without CIP (0 to 12 days), limiting useful comparisons.

Identified pattern of increased levels of inflammatory markers and ICU-AW

Our results suggest that IL-6, IL-8, IL-10 and fractalkine may be involved in the pathogenesis of ICU-AW. The both pro- and anti-inflammatory acting cytokine IL-6, pro-inflammatory acting cytokine IL-8, and anti-inflammatory cytokine IL-10 are important factors in the onset of the systemic inflammatory and anti-inflammatory responses. They play an important role in the disbalanced inflammatory response as is seen in sepsis and MODS.[22] Fractalkine (CX3CL1) is a recently discovered inflammatory mediator, which can be expressed in several tissues, including skeletal muscle and neurons.[23] It can act both as adhesion molecule and as a soluble chemokine and is correlated with disease severity in sepsis patients in the ICU.[24] IL-6, IL-8, IL-10 and fractalkine are described as prognostic biomarkers in sepsis: IL-6, IL-10 and fractalkine can distinguish between survivors and non-survivors at day 28 and IL-8 has been used for the prediction of MODS.[25,26] As these markers can predict severity of sepsis, an association with ICU-AW, a severe complication of sepsis, would not be surprising.

In vitro and in vivo experiments have shown that cytokines are implicated in muscle damage[27,28] and that the systemic inflammatory response results in local

production of cytokines and acute phase proteins in muscle.[29-31] IL-6 and fractalkine act as chemoattractants, recruiting cytokine producing leukocytes to the muscle, leading to proteolysis, myocyte degeneration, and muscle atrophy.[27] IL-10 is an anti-inflammatory protein and can inhibit IL-6. It may not be the absolute values of pro-inflammatory cytokines that produce muscle damage, but the imbalance between pro-and anti-inflammatory cytokines.[27] Capillary leakage and hypoxia causing muscle and nerve damage are an indirect consequence of activated inflammatory markers.[9]

Interestingly, IL-6 and IL-8, along with IL-15, can be expressed by and also released from skeletal muscle, they are so-called myokines.[32] Release of these myokines is mainly described after exercise, where it is believed to play a protective role in the local signaling and regulation of inflammatory markers.[33] It might be possible that, in critical illness, release of myokines, especially IL-6, may contribute to systemic inflammation, perpetuating and disturbing the systemic inflammatory response, possibly adding to multiple organ failure and muscle and nerve damage.

The other markers and patterns we investigated in this study did not seem to be different in patients with and without ICU-AW. This was possibly limited by the fact that many measurements of IL-1β, IL-13, TNFα, IFNγ and GM-CSF were below the detection limit. In PLS-DA, we found that IL-1β and IFNγ on day 0 were also important factors, but this was not seen in PLS-DA of day 2 and 4. In contrast, in multivariable logistic regression with PCs, ICU-AW was associated with lower values of IL-1, IL-13 and TNFα (PC2), but this PC was not significant in pooled analysis of imputed datasets.

Sepsis is associated with increased expression of vascular adhesion molecules on endothelium and increased shedding of these molecules, leading to accumulation of soluble forms in the blood.[34] Increased shedding seems to diminish inflammation and high levels of soluble adhesion molecules are associated with better outcomes in sepsis.[35] In accordance with this, we found that PC3 representing soluble vascular adhesion molecules gave a lower risk of ICU-AW, although this was not significant in pooled data from imputed datasets and should therefore be interpreted with caution.

Strengths and limitations

The large sample size and serial collection of blood samples are the main strengths of this study. Taking the complexity of the systemic inflammatory response into consideration, we are the first to investigate patterns of inflammatory markers using statistical procedures like principal component analysis. By including also patients without sepsis and performing stratified analysis we showed that the association found was not restricted to patients with sepsis. ICU-AW was systematically diagnosed, using MMT. Even though MMT has its limitations, it is the most reliable

test and the experts' recommended method to diagnose ICU-AW.[18,19] We diagnosed ICUAW at the earliest time point possible. This leads to a variable time window to the diagnosis of ICU-AW, but it is our experience that by choosing a set time for MMT assessment there is an increased risk for missing patients without ICU-AW because they will in general be discharged earlier from the ICU.

This study also has limitations. First of all, we did not perform a power calculation, because data from previous studies did not allow a reliable sample size calculation. Secondly, corticosteroid use on the ICU, APACHE IV score and maximal SOFA score were higher in the group with ICU-AW. Although we included these factors (except APACHE IV, because of collinearity) as confounders in our statistical analysis, we cannot completely rule out some residual confounding.

Furthermore, we described an association between ICU-AW and increased inflammatory markers, but no causal relations can be deduced from this observational study.

Finally, serial measurements of inflammatory markers early after ICU admission restrict our study to statements about inflammation in the first four days after ICU admission. The time of onset of ICU-AW in our patients is unknown, because muscle strength can only be evaluated when patients are awake and attentive, in our study after a median of nine days. However, ICU-AW is assumed to develop early, since electrophysiological studies have described abnormalities within three days after ICU admission.[36] The use of electrophysiological measurements in our study could have been of additional value, but this was not possible in our study set-up.

Recommendations for future research

Our recommendation for future research is to explore IL-6, IL-8, IL-10 and fractalkine and their possible pathophysiological role in ICU-AW in animal or laboratory studies. It should be further investigated if these inflammatory markers play a causal role in the development of ICU-AW and whether this is via a direct or indirect pathway. It would also be interesting to investigate whether these markers differ between patients with a polyneuropathy and patients with a myopathy, although most patients with ICU-AW have a combined polyneuropathy and myopathy.[2]

The focus should not be on the individual markers but on their combined functions as these markers interact with each other in complex inflammatory networks. Further unraveling of the involved pathways may open a way to modulate the inflammatory response and possibly prevent ICU-AW.

CONCLUSIONS

Systemic inflammation is increased in the first four days after ICU admission in critically ill patients who develop ICU-AW compared to critically ill patients who do not develop ICU-AW. ICU-AW is independently associated with increased levels of IL-6, IL-8, IL-10 and fractalkine. These four markers may be important in the pathophysiology of ICU-AW.

ACKNOWLEDGEMENTS

CONFLICTS OF INTEREST AND SOURCE OF FUNDING

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DATA SUPPLEMENT

ADDITION TO METHODS

Inflammatory marker assays

Measurements were done using FlexSet cytometric bead arrays (BD Bioscience, San Jose, CA) using FACS Calibur (Becton Dickenson, Franklin Lakes, NJ, USA). Several quality assurance measures were taken. Before every run, the FACS apparatus was calibrated with calibration beads. Reagents were from the same batch. All samples were randomly put on the plates (independent of time point, presence of ICU-AW etc.). Furthermore, the analysts who performed the FACS analysis were blinded for the time point, outcome and all other patient characteristics. When concentrations were below or above the detection limit of the assay, we used the lowest or highest threshold as value, respectively.

Statistical analysis

Plasma levels of inflammatory markers were logarithmically transformed (using the natural logarithm) to approximate normality before analysis.

Depending on the distribution of the data, mean values are presented with standard deviation (±SD), median values with interquartile range (IQR) and proportions with total numbers and percentages. Differences between proportions were assessed using chi-square or Fisher's exact test, differences between normally distributed variables using Welch's t-test and differences between non-normally distributed continuous variables using Mann-Whitney U test.

A P-value <0.05 was considered statistically significant. Outcomes are presented with 95% confidence intervals (CI). In univariable analysis of inflammatory markers, Bonferroni correction was used because of multiple testing (cut-off for statistical significance p<0.004).

Analyses were done using R (version: 3.0.2, R Foundation for Statistical Computing, Vienna, Austria).

Principal component regression analysis

With principal component analysis (PCA), we converted our set of inflammatory markers (all time points together) into a set of uncorrelated variables called principal components (PCs) and reduced the amount of variables to put in a multivariable logistic regression model. The inflammatory markers were centered and scaled for PCA. To determine the number of components to retain, we used Horn's parallel analysis.[1] Retained principal components were put in a multivariable logistic regression model together with a priori selected possible confounders for ICU-AW.

Varimax rotation was used to improve interpretation of component loadings. Variables with a loading above the cut-off point 0.30 were considered to be the dominant variables in a component.

Partial least squares-discriminant analysis (PLS-DA)

Variables with a Variable Importance in Projection (VIP) score >1 were considered important in the PLS-DA model.[3] We also did a stratified analysis for patients with and patients without sepsis, to investigate if a difference in systemic inflammation might be explained by sepsis alone.

Mixed-effects models

We used linear mixed-effects models with the inflammatory marker as the dependent variable (outcome) and ICU-AW a independent variable, because interpretation of regression coefficients of mixed-effects logistic regression models is challenging. To understand the relationship between ICU-AW and the inflammatory marker, it is easier to interpret the regression coefficients in a linear mixed-effects model as this gives the association between the presence of the syndrome ICU-AW and the concentration of the inflammatory marker.

First, a model was made including the previously mentioned confounders as fixed effects, but without the variable ICU-AW. Patient was added as a random factor. Then, the variable ICU-AW was added to the model. If adding ICU-AW decreased the Akaike Information Criterion (AIC) >2 points, the model with ICU-AW was considered to be a better model.[4]

Missing values

Missing values were considered missing at random and were imputed using multivariate imputations by chained equations (10 iterations of 10 imputations) using all available variables to predict the missing values. Each of the datasets was analyzed and if possible results were pooled using Rubin's rules which accounts for the uncertainty associated with imputed values.[5]

Sample size

The sample size of this observational exploratory study was chosen on pragmatic grounds. We aimed to include 100 patients per group. With a suspected ICU-AW prevalence of 50% in our population, we therefore intended to include 200 patients in this study. Previous data on inflammatory markers in ICU-AW did not allow for a formal sample size calculation.

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eTable 1. Distributions of inflammatory markers
Log transformed distributions of inflammatory markers

Inflammatory marker	ICU-AW 264 plasma samples	No ICU-AW 275 plasma samples	P-value
IL-1β (median pg/ml [IQR])	1.3 [0.3; 4.2]	0.3 [0.3; 2.4]	<0.001*
IL-6 (median pg/ml [IQR])	118.5 [35.2; 603.3]	48.0 [18.8; 156.9]	<0.001*
IL-8 (median pg/ml [IQR])	157.2 [60.7; 517.7]	57.2 [23.9; 143.7]	<0.001*
IL-10 (median pg/ml [IQR])	19.7 [7.1; 66.1]	7.0 [2.7; 20.0]	<0.001*
IL-13 (median pg/ml [IQR])	0.2 [0.2; 0.2]	0.2 [0.2; 0.2]	0.784
TNFα (median pg/ml [IQR])	0.3 [0.3; 1.3]	0.3 [0.3; 0.8]	0.015
IFNγ (median pg/ml [IQR])	1.9 [0.1; 27.0]	0.1 [0.1; 9.0]	0.003*
GM-CSF (median pg/ml [IQR])	0.5 [0.5; 0.5]	0.5 [0.5; 0.5]	0.602
Fractalkine (median pg/ml [IQR])	48.7 [24.8; 110.1]	20.8 [12.0; 50.8]	<0.001*
sICAM-1 (median ng/ml [IQR])	223.3 [109.8; 349.7]	144.1 [87.9; 259.0]	<0.001*
sP-Selectin (median ng/ml [IQR])	29.9 [14.1; 62.9]	26.5 [15.1; 46.6]	0.156
sE-Selectin (median ng/ml [IQR])	7.9 [3.7; 17.4]	8.6 [3.8; 21.4]	0.361

*significantly different after Bonferroni correction for multiple testing

eTable 2. Number of measurements per inflammatory marker and number and percentage below and above detection limit.

Inflammatory mediator	Number of measurements	Below detection limit (%)	Above detection limit (%)
IL1	539	234 (43.4)	0
IL6	539	5 (0.9)	0
IL8	539	1 (0.2)	1 (0.002)
IL10	539	23 (4.3)	0
IL13	539	388 (72.0)	0
TNFa	539	348 (64.6)	0
IFNg	539	267 (49.5)	0
GM-CSF	539	445 (82.6)	0
Fractalkine	539	86 (16.0)	0
sICAM-1	538	0	9 (1.7)
sP-Selectin	538	0	0
sE-Selectin	538	0	0

Table 3. Loadings of principal components 1 to 3 after varimax rotation.

	PC1	PC2	PC3
IL-1β	0.112	**-0.444**	
IL-6	**0.502**		
IL-8	**0.546**		
IL-10	**0.477**		
IL-13	-0.173	**-0.525**	0.119
TNFα		**-0.596**	
IFNγ	0.180	-0.262	
GM-CSF		-0.289	-0.178
Fractalkine	**0.326**		
sICAM-1	0.127		**0.508**
sP-Selectin	-0.113		**0.541**
sE-Selectin			**0.627**

In bold the main loadings with cut-off >0.30.
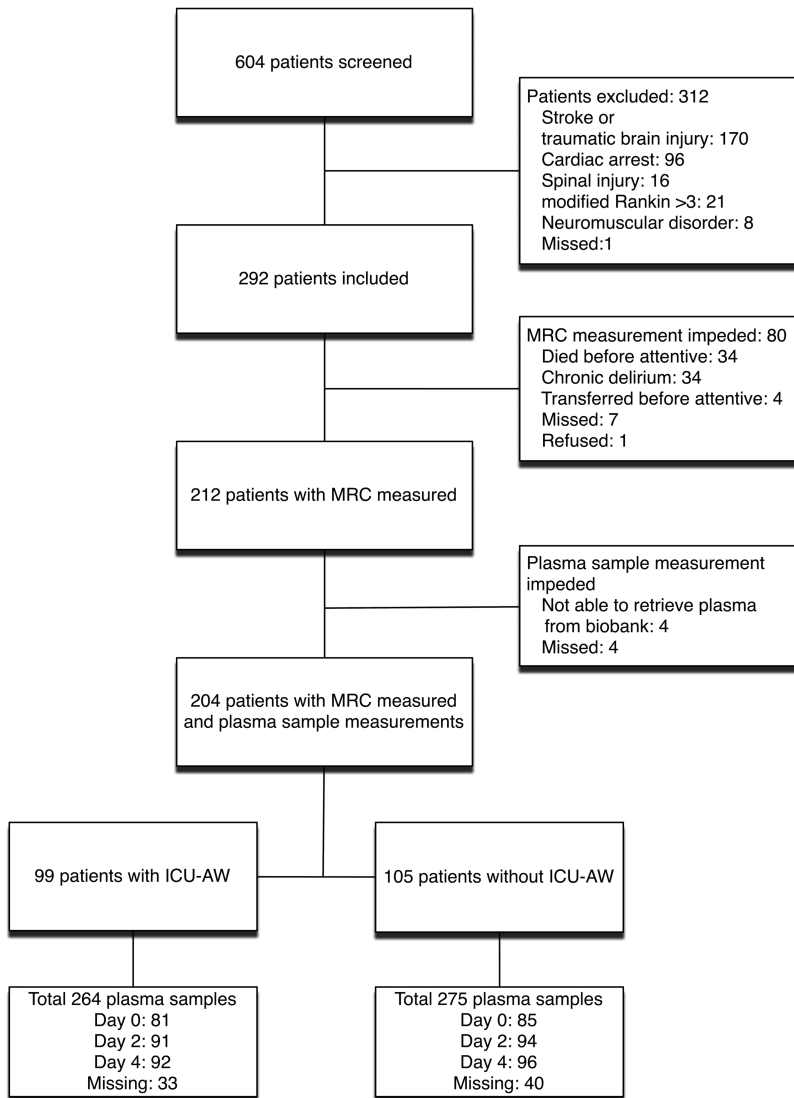
eFigure 1. Flowchart of screened and included patients.
ICU-AW=ICU-acquired weakness; MRC=muscle strength as assessed with Medical Research Council scale.
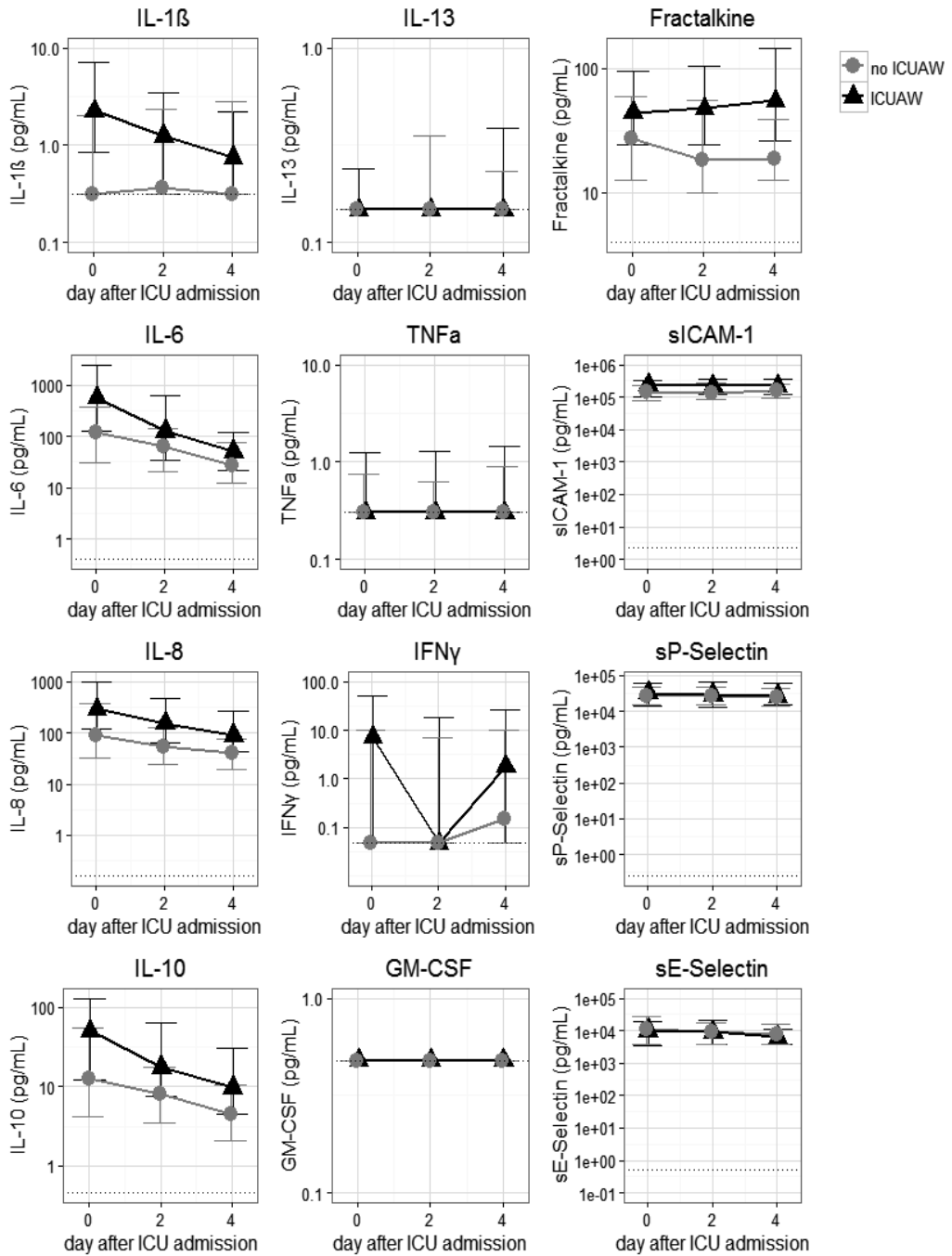
eFigure 2. Graphs of inflammatory markers over time
Values are plotted with median and interquartile range.

A **B** **C**

eFigure 3. Course of PC1 to PC 3 over time
Course of PC1(panel A); PC 2 (panel B) and PC 3 (panel C) over time. P-values are derived from linear mixed-effects models with confounders included.
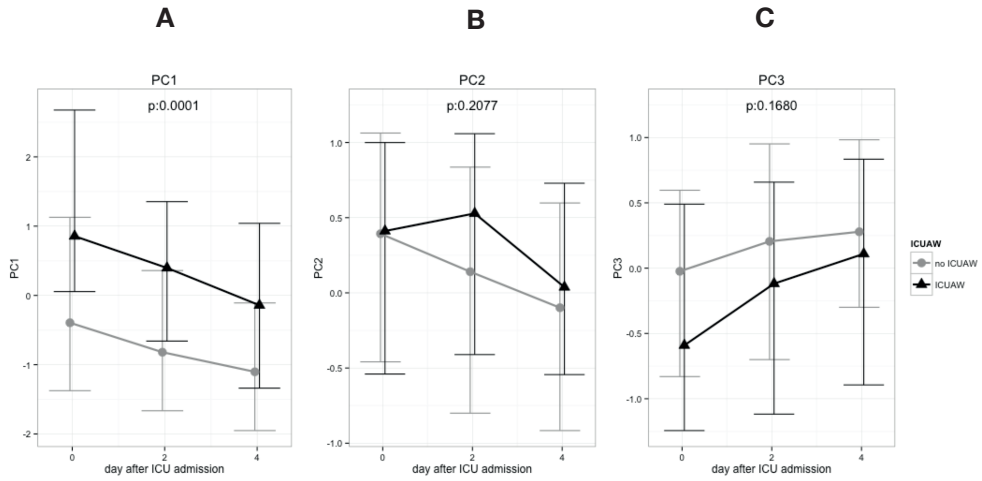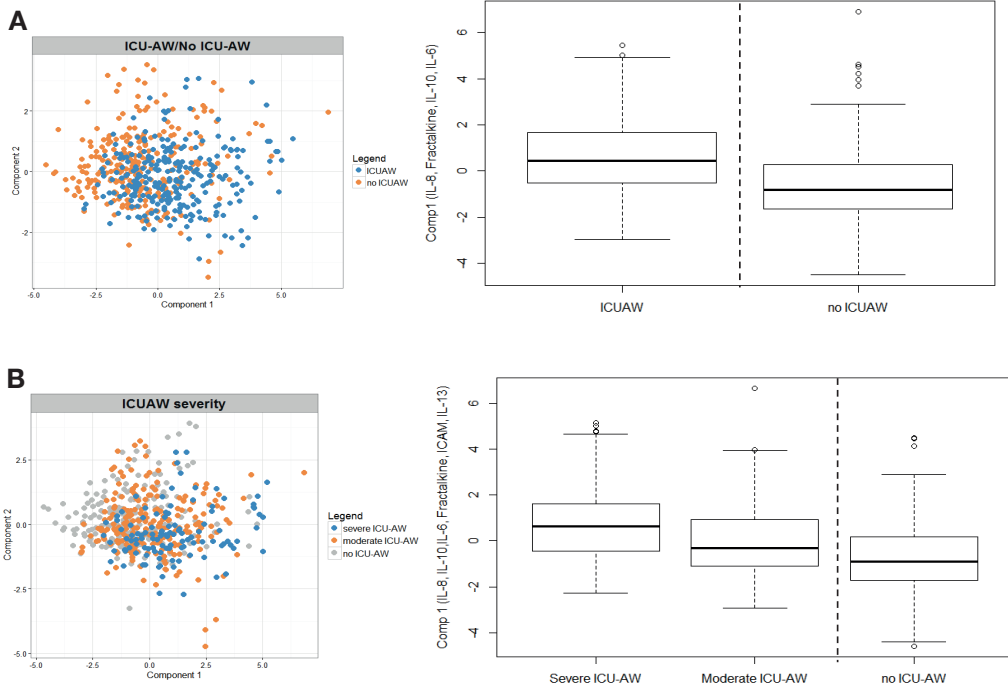

eFigure 4. Scores plots and distributions of component 1 of PLS-DA
Scores plot of first 2 components of PLS-DA and distributions of component 1 of patients with and without ICU-AW (A) and different ICU-AW severity (B) (with severe ICU-AW defined as an average MRC<3). In the analysis of patients with or without ICU-AW the most important variables in component 1 were IL-8; Fractalkine; IL-10 and IL-6. In the analysis of ICU-AW severity the most important variables in component 1 were IL-8; IL-10; IL-6; Fractalkine; ICAM and IL-13.
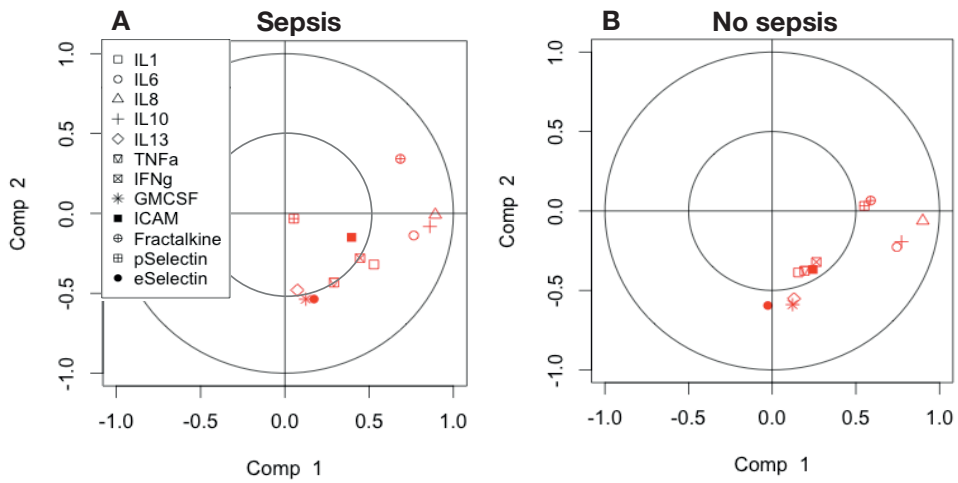
eFigure 5. PLS-DA loading plot for patients stratified by sepsis
PLS-DA loading plot for patients with sepsis (n=438 measurements; panel A) and without sepsis (n=100 measurements; panel B).

CHAPTER 5

No association between systemic complement activation and intensive care unit-acquired weakness

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ABSTRACT

Background

The main risk factors for intensive care unit-acquired weakness (ICU-AW) are sepsis, the systemic inflammatory response syndrome and multiple organ dysfunction. These risk factors are associated with systemic complement activation. We hypothesized that critically ill patients who develop ICU-AW have increased systemic complement activation compared to critically ill patients who do not develop ICU-AW.

Methods

Complement activation products C3b/c, C4b/c and C5a were measured in plasma of ICU patients with mechanical ventilation for ≥48 hours. Samples were collected at admission to the ICU and for six consecutive days. ICU-AW was defined by a mean Medical Research Council score <4. We compared the level of complement activation products between patients who did and who did not develop ICU-AW.

Results

Muscle strength measurements and complement assays were available in 27 ICU patients, of whom 13 patients developed ICU-AW. Increased levels of C4b/c were seen in all patients. Neither admission levels, nor maximum, minimum and mean levels of complement activation products were different between patients who did and did not develop ICU-AW.

Conclusions

Complement activation is seen in critically ill patients, but is not different between patients who did and who did not develop ICU-AW.

INTRODUCTION

The pathogenesis of intensive care unit-acquired weakness (ICU-AW) is probably multi-factorial.[1] The main risk factors are sepsis, systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS).[1] Activation of the complement system plays an important role in these risk factors and is associated with increased occurrence of shock and fatal outcomes in sepsis.[2-4] Complement activation also plays a role in the pathogenesis of acute inflammatory polyneuropathies and myopathies.[5-7] Thus, muscle and nerve damage in ICU-AW might also be complement-mediated.

Complement activation may lead to ICU-AW by anaphylatoxins (C3a, C4a, C5a), which can induce unbalanced systemic and local inflammatory responses, leading to MODS[8,9] and probably to 'failure' of muscles and nerves. C5a can also increase vascular permeability, leading to tissue edema and possibly nerve and muscle tissue damage.[9] The final pathway of complement activation results in the membrane attack complex (MAC), causing direct cell damage.[9] MAC depositions have been found in muscles of patients with ICU-AW.[10-12]

In this pilot study, we tested the hypothesis that patients who develop ICU-AW have increased systemic complement activation compared to critically ill patients who do not develop ICU-AW.

MATERIALS AND METHODS

This was a sub-study of a prospective observational cohort study (BASIC study, Biomarker Analysis in Septic Intensive Care patients), performed on the mixed medical-surgical ICU of the Academic Medical Center Amsterdam. The institutional review board approved the BASIC study protocol (number NL34294.018.10). Informed consent from patients or their legal representatives was obtained before study participation.

Patients newly admitted to the ICU having sepsis or SIRS (Bone criteria[13]) mechanically ventilated for ≥48 hours, and in whom muscle strength assessment was performed were eligible for inclusion. Exclusion criteria included antibiotic treatment for >48 hours, expected ICU stay <24 hours, no informed consent within 24 hours after ICU admission, pre-existing poor functional status (Modified Rankin score ≥4[14]) and any central nervous system disorder, spinal cord injury or neuromuscular disorder as reason for ICU admission.

Blood samples were collected as soon as possible after ICU admission, and thereafter daily (~3:00 PM) for six consecutive days. Blood was collected in vacutainer tubes, containing an inhibitor mix (with final concentrations of 10 mM benzamidine, 100 µg/ml soy bean trypsin inhibitor and 10 mM ethylene-diamine-tetra-acid) to prevent *in vitro* complement activation. Samples were centrifuged

(1.500 x g, 15 min, room temperature) within one hour after collection and plasma was stored in aliquots at -80°C until assayed.

To determine complement activation of the common pathway and initial classical/lectin or alternative pathway, plasma levels of complement activation products C3b/c and C4b/c were measured using previously described enzyme-linked immunosorbent assays (ELISAs) (Sanquin, Amsterdam, The Netherlands).[15,16] These ELISAs do not distinguish C3b from C3bi and C3c, and C4b from C4bi and C4c and are therefore referred to as C3b/c and C4b/c. The normal reference values (from local healthy controls) are <57 nmol/L for C3b/c and <8 nmol/L for C4b/c.

Further downstream complement activation was assessed by measuring levels of C5a (no reference value available), using a commercial ELISA kit (MicroVue, Quidel, San Diego, USA).

All measurements were done batch-wise and in duplo. Samples were analyzed blinded to all patients' data. Measurements with a coefficient of variation (CV) value of >30% were excluded from the analysis. The ELISA was successfully performed (CV of <30%) in 99% of C3b/c measurements, 98% of C4b/c measurements and 100% of C5a measurements. The lower limit of detection (determined by the mean of blanks plus 3 times the standard deviation of the blanks) for the C3b/c, C4b/c and C5a assays were 0.001 nmol/L, 0.002 nmol/L and 0.004 ng/mL, respectively.

Manual muscle strength was assessed as soon as patients were awake and attentive. Using the Medical Research Council (MRC) scale, six muscle groups were tested, bilaterally. ICU-AW was defined by a mean MRC score <4.[17,18]

The following clinical characteristics were collected: age, gender, admission reason, presence of sepsis at admission, length of stay on the ICU, number of days with mechanical ventilation, days from admission to muscle strength assessment, ICU mortality, Acute Physiology and Chronic Health Evaluation IV (APACHE IV) score and Sequential Organ Failure Assessment (SOFA) scores at days of blood sampling.

This study is an exploratory pilot study. Therefore, no formal power calculation was performed.

Mean values are presented with standard deviation (±SD), median values with interquartile range (IQR) and proportions with total numbers and percentages. Differences between proportions were assessed using Fisher's exact test, between normally distributed continuous variables using Welch's t-test and between non-normally distributed continuous variables using Mann-Whitney U test.

To assess our primary endpoint, the difference between systemic complement activation and the presence of ICU-AW, and to account for repeated measurements we used summary statistics, which included: admission complement levels, maximum, minimum and mean values per patient during the first seven days in ICU.

A p-value <0.05 was considered statistically significant (p< 0.004 after Bonferroni correction). Analyses were done using R (version: 3.0.2).

RESULTS

Data and plasma samples of 27 patients were available; 13 patients who developed ICU-AW and 14 patients who did not develop ICU-AW. Patient characteristics are presented in table 1. A total of 167 plasma samples were analysed (median of seven samples/patient). The median time from ICU admission to the first sample was 15.8 hours (IQR 12.8-22.5) in the ICU-AW group versus 17.3 hours (IQR 14.1-22.2) in the no ICU-AW group (p: 0.74).

Levels of C3b/c, C4b/c and C5a fluctuated considerably in individual patients during the first seven days in ICU. Median levels and interquartile range of C3b/c, C4b/c and C5a at each time point are presented in figure 1.

There was no difference in admission, maximum, minimum or mean levels of C3b/c, C4b/c or C5a between patients who developed and did not develop ICU-AW (table 2).

Table 1. Patient characteristics

	ICU-AW n:13	no ICU-AW n:14	p-value
Age, median years (IQR)	72.0 (63-76)	58.0 (43.5-64.8)	0.01
Males, n (%)	7 (53.8)	6 (42.9)	0.71
Sepsis at admission, n (%)	12 (92.3)	10 (71.4)	0.33
Admission reason			0.76
medical, n (%)	9 (69.2)	10 (71.4)	
planned surgical, n (%)	1 (7.7)	2 (14.3)	
emergency surgical, n (%)	3 (23.1)	2 (14.3)	
APACHE IV score, mean (sd)	91.5 (28.9)	76.4 (34.2)	0.23
Maximal SOFA score on day of blood sample, median (IQR)	13.0 (9.0-14.0)	8.0 (5.3-12.3)	0.12
Number of blood samples per patient, median (IQR)	7.0 (6.0-7.0)	7.0 (6.0-7.0)	0.74
Mean MRC score, median (IQR)	2.8 (1.3-2.9)	4.5 (4.1-4.9)	
Day of MRC score, median (IQR)	7.0 (6.0-11.0)	7.0 (6.0-9.0)	0.88
Days with MV, median (IQR)	10.0 (6.0-13.0)	5.0 (4.3-7.8)	0.07
LOS ICU, median (IQR)	13.0 (8.0-17.0)	8.0 (7.0-11.0)	0.13
Died on the ICU, n (%)	5 (38.5)	0 (0.0)	0.02

ICU-AW=intensive care unit-acquired weakness; IQR=interquartile range; APACHE=Acute Physiology and Chronic Health Evaluation; SOFA=sequential organ failure assessment; MRC=medical research council; MV=mechanical ventilation; LOS=length of stay; ICU=intensive care unit.

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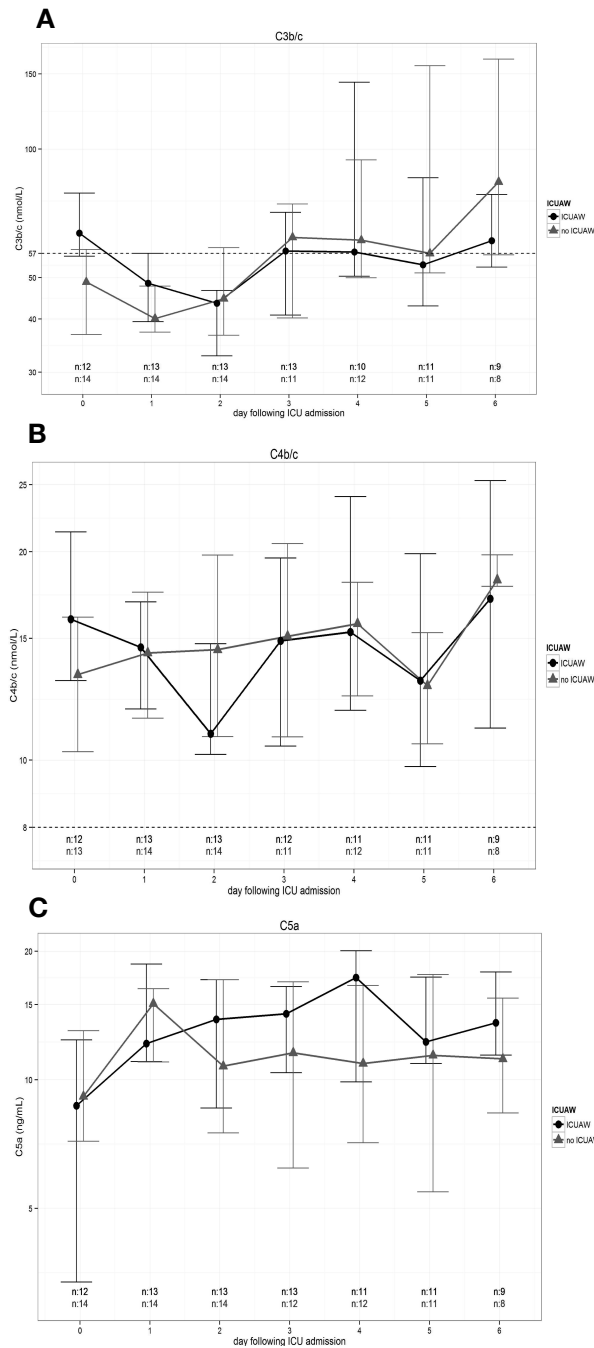
Figure 1. Levels of C3b/c, C4b/c and C5a in patients who developed and did not develop ICU-AW

Levels of C3b/c (panel A), C4b/c (panel B) and C5a (panel C) at admission (day 0) and six consecutive ICU days in patients who developed and who did not develop ICU-AW. Data are presented as median with interquartile range for each time point and numbers below the lines represent the number of samples of patients with ICU-AW (black) and without ICU-AW (grey). Dotted lines (in A and B) represent the reference values.

Table 2. Admission, maximum, minimum and mean levels of C3b/c, C4b/c and C5a.
Admission levels, maximum levels per patient, minimum levels per patient and mean levels per patient of complement activation products in the first 7 days in ICU in patients who developed and who did not develop ICU-AW. Levels are presented as median and interquartile range (C3b/c and C4b/c in nmol/L and C5a in ng/mL).

	ICU-AW n:13	No ICU-AW n:14	p-value
C3b/c admission levels	63.5 (56.1-78.8)	48.8 (36.8-58.2)	0.11
C3b/c max levels	107.0 (82.4-201.4)	90.0 (72.6-196.2)	0.55
C3b/c min levels	35.9 (32.3-39.8)	33.8 (26.5-38.6)	0.55
C3b/c mean levels	62.8 (56.9-94.4)	62.0 (50.4-95.4)	0.72
C4b/c admission levels	16.0 (13.0-21.4)	13.3 (10.3-16.1)	0.17
C4b/c max levels	23.1 (17.1-34.0)	24.0 (19.3-38.4)	0.37
C4b/c min levels	9.9 (7.0-10.9)	9.6 (6.6-11.2)	0.87
C4b/c mean levels	15.3 (12.0-19.9)	16.4 (13.3-23.6)	0.62
C5a admission levels	8.7 (3.4-12.4)	9.1 (7.2-13.0)	0.53
C5a max levels	18.6 (11.6-35.7)	16.6 (11.5-19.1)	0.58
C5a min levels	8.5 (3.3-11.0)	7.4 (4.4-9.9)	0.83
C5a mean levels	13.1 (9.7-18.8)	11.9 (7.7-16.0)	0.65

ICU-AW=intensive care unit-acquired weakness.

DISCUSSION

This pilot study shows no difference in systemic complement activation in the first seven days after ICU admission between patients who did and who did not develop ICU-AW.

All patients showed increased complement activation, as shown by C4b/c levels of nearly twice the reference value. The in- and exclusion criteria were rather strict and it is likely that we have selected a severely ill subpopulation with a high inflammatory state at admission. The severity of illness in the ICU-AW and no ICUAW group were comparable; both groups had high APACHE IV and SOFA scores.

Complement levels were lower than previously described in a study with patients with severe sepsis and septic shock[19], possibly due to a different case mix: not all patients in our cohort had sepsis, and patients might have died earlier in other cohorts, before muscle strength could have been measured.

Although we did not find a difference in complement activation, activated complement can still play a role in the pathophysiology of ICU-AW in the presence

of another yet unknown factor, for example expression of membrane complement regulatory proteins (the sensitivity to complement-mediated injury).[20]

The difference in systemic complement levels between patients with and without development of ICU-AW has never been studied before. Previously, no correlation has been found between plasma C3 and C4 levels and compound muscle action potential (CMAP) amplitudes of three nerves in ICU patients, but muscle strength was not measured in this study.[21]

The use of daily measurements is a strength of this study because it enabled us to investigate the time course of complement activation and to use summary statistics, such as maximum levels.

This study has some limitations. The sample size of this pilot study was small, limiting the robustness of our results. Furthermore, it was impossible to determine the exact moment at which the inflammatory process was triggered in individual patients, since the onset of this process may take place before ICU admission.[22] As complement activation occurs very early in the inflammatory response, peaks of complement activation within the first hours after ICU admission may be missed. Hemodilution may also have decreased complement levels[15], but this is a difficult factor to correct for. Furthermore, it can be debated whether plasma levels of complement activation products, indicating systemic activation, adequately reflect the levels in muscle or nerve tissue, since complement can also be activated locally.[12]

Muscle strength assessment by MRC is the recommended test for diagnosing ICU-AW.[18,23] A diagnosis of ICU-AW by MRC is often delayed due to impaired consciousness. Therefore, the moment at which ICU-AW developed is unknown. ICU-AW may develop very early, because electrophysiological signs of ICU-AW have been found already within three days after ICU admission.[24] We did not perform electrophysiological investigations. Therefore it is unknown if patients had electrophysiological alterations at the time the blood samples were taken. Finally, muscle strength might have returned to normal at the time patients woke up, because early detected electrophysiological alterations can be rapidly reversible.[25]

CONCLUSION

This pilot study shows that systemic complement levels are not different between patients with or without ICU-AW.

ACKNOWLEDGEMENTS

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PART II

INTENSIVE CARE UNIT-

ACQUIRED WEAKNESS IN

EXPERIMENTAL MOUSE MODELS

CHAPTER 6

Assessment of intensive care unit-acquired weakness in young and old mice: an *E. coli* septic peritonitis model

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ABSTRACT

Introduction

There are few reports of *in vivo* muscle strength measurements in animal models of ICU-acquired weakness (ICU-AW). In this study we investigated whether the *Escherichia coli* (*E. coli*) septic peritonitis mouse model may serve as an ICU-AW model using *in vivo* strength measurements and myosin-actin assays, and whether development of ICU-AW is age-dependent in this model.

Methods

Young and old mice were injected intraperitoneally with *E. coli* and treated with ceftriaxone. Forelimb grip strength was measured at multiple time points, and myosin-actin ratio in muscle was determined.

Results

E. coli administration was not associated with grip strength decrease, neither in young nor in old mice. In old mice, the myosin-actin ratio was lower in *E. coli* mice at t=48 and higher at t=72 hours compared to controls.

Conclusion

This *E. coli* septic peritonitis mouse model did not induce decreased grip strength. In its current form, it seems unsuitable as a model for ICU-AW.

INTRODUCTION

Intensive care unit-acquired weakness (ICU-AW) is a frequent complication of critical illness, which causes long-term impairments in physical function.[1,2] ICU-AW is caused by critical illness myopathy (CIM), critical illness polyneuropathy (CIP), or a combination of both (CINM).[1] The most prominent characteristic and prerequisite for the diagnosis of ICU-AW is decreased muscle strength as measured by muscle strength testing in awake and responsive patients.[1]

Several sepsis animal models have been used to study ICU-AW, as sepsis is the main risk factor for development of ICU-AW.[3] However, the models used so far have severe limitations. First, *in vivo* quantitative muscle strength measurements in awake mice have been performed infrequently. Instead, other markers for muscle function have been used, like electromyography or contractility measurements, which require anesthesia.[4-6] Moreover, animal models often use a short period of sepsis, which is not comparable to the chronic sepsis seen in ICU-AW patients.[7] Models of long-lasting sepsis, such as the porcine model of acute quadriplegic myopathy, are very expensive and time-consuming.[5] Finally, animals used in experiments are often young, whereas increasing age has been described as a risk factor for ICU-AW.[8,9] Therefore, a new animal model to study ICU-AW is needed in order to ensure more reliable translation of results from animal experiments to the bedside. In such a new model, animals with sepsis should show a clinically significant decline in muscle strength over time, and animals of older age should be studied. Since a selective loss of myosin filaments is seen in patients with ICU-AW caused by CIM or CINM, the myosin-actin ratio can also be used to characterize a new animal model.[10]

Intra-abdominal infections are an important cause of human sepsis, and *Escherichia coli* (*E. coli*) bacteria are frequently involved.[11] In this study we used the *E. coli* septic peritonitis mouse model, a well-established animal model for sepsis.[12,13]

The objectives of this study were to investigate: (1) whether the *E. coli* septic peritonitis model induces ICU-AW using both *in vivo* strength measurements and *in vitro* myosin-actin assays and (2) whether development of decline in muscle strength is age-dependent in this model.

MATERIALS AND METHODS

Animals and ethics statement

A total of 108 male, specified pathogen-free, C57BL/6J mice were obtained from Charles River; 54 mice were 8 weeks old (young mice), and 54 mice were 13 months old (old mice). Mice were housed in groups of 5 to 6 in individually ventilated cages for at least 2 weeks before the start of the experiment. All experiments conformed to the Dutch Experiments on Animals Act for the care and use of animals and were

approved by the Institutional Animal Care and Use Committee of the Academic Medical Center, Amsterdam, the Netherlands (permit number: 102639). Food and water were available *ad libitum,* and a 12:12 hour light-dark cycle was retained. This manuscript was drafted in accordance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines.[14]

Induction of E. coli peritonitis and treatment with antibiotics

E. coli (O18:K1) was cultured in Luria-Bertani medium at 37°C to mid-log phase in 1 hour, 45 minutes. The amount of bacteria in the culture was estimated by measuring the A600 in a spectrophotometer. Viable *E. coli* were harvested by centrifugation at 3000 rpm for 10 minutes and washed twice with pyrogen-free sterile isotonic saline. The bacteria were diluted to a final concentration of $1x10^4$ colony forming units (CFU) /200 µl (range $0.8x10^4$ - $1.2x10^4$ CFU) in pyrogen-free sterile isotonic saline. Experiments were performed in 2 different rounds (first round, 22 young mice and 22 old mice; second round, 32 young mice and 32 old mice). Serial 10-fold dilutions of the bacterial inoculum were plated on blood agar plates and incubated overnight at 37°C to verify the amount of viable bacteria injected.

Without antibiotic treatment, 80% of mice in this model die within 48 hours.[13] To improve survival and prolong the duration of the model, enabling repeated muscle strength measurements over time, mice received antibiotic treatment with ceftriaxone (Fresenius Kabi, Den Bosch, the Netherlands) 10µl/g body weight (20mg/kg).

Experimental procedure

Young and old mice were assigned to 3 groups (each cage was randomly assigned to 1 of the groups): *E. coli* and antibiotics (E+A group, 30 young mice and 30 old mice), control and antibiotics (C+A group, 12 young mice and 12 old mice), and control (C group, 12 young mice and 12 old mice) (table 1).

At the start of the experiment (t=0), E+A mice were injected intraperitoneally with 200 µl of the bacterial inoculum; the C+A group and C group were injected intraperitoneally with 200 µl of pyrogen-free sterile isotonic saline. At t=12 and t=24 hours after injection, E+A and C+A mice received an intraperitoneal injection with ceftriaxone. At these time points mice of the C group received saline (10µl/g body weight). Body weight was measured at baseline, at t=12, t=24, t=48, and t=72 hours. Half of the animals in each group were euthanized at t=48 hours and the other half at t=72 hours. Animals were euthanized by an intraperitoneal injection of a mix of ketamine 190 mg/kg and medetomidine 0.3 mg/kg followed by heart puncture and blood aspiration. Blood, spleen, and liver were harvested for determination of bacterial outgrowth. Tibialis anterior muscles were removed, snap frozen in liquid nitrogen, and stored at -80ºC.

Table 1. Experimental groups

Group	t=0	t=12	t=24	t=48	t=72
E+A group	*E. coli*	Ceftriaxone	Ceftriaxone	Sacrificed	Sacrificed n=15 young
n=30 young				n=15 young	n=14 old*
n=30 old				n=15 old	
C+A group	Saline	Ceftriaxone	Ceftriaxone	Sacrificed	Sacrificed
n=12 young				n=6 young	n=6 young
n=12 old				n=6 old	n=6 old
C group	Saline	Saline	Saline	Sacrificed	Sacrificed
n=12 young				n=6 young	n=6 young
n=12 old				n=6 old	n=6 old

*1 old mouse from the E+A group died before the end of the experiment.
E+A=*E. coli* and antibiotics; C+A=control and antibiotics; C=control.

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Grip strength testing

At t=0, t=12, t=24, and t=48 (at t=48 half of the animals), forelimb grip strength was measured using a grip strength meter with metal grid (Bioseb, France). Mice were held at the base of the tail above the top of the grid. After holding the grid, they were pulled backwards horizontally until the grip was released. Maximal force developed by the animal was recorded by the grip strength meter. At each time point, 3 grip strength measurements were taken, and the average result was used for analysis. Grip strength was normalized for concomitant body weight.[15]

Bacterial outgrowth

Spleen and liver were homogenized in 4 volumes of sterile saline with a tissue homogenizer. Serial 10-fold dilutions of blood, liver, and spleen homogenates were plated on blood agar plates, and bacteria were allowed to grow overnight at 37°C.

Whole muscle homogenate

Frozen muscle samples of E+A and C+A mice were pulverized with a pre-cooled mortar and hammer. The pulverized muscle was then suspended in a vial with 500 µl of lysis buffer (5 mM Sigma 7-9, 50 mM NaF, 2 mM Na_3VO_4, 2 mM EGTA, distilled water, with protease inhibitor mix (aprotinin, leupeptin and pepsatin in Tris-HCL solution), and DTT. Tissue was homogenized by use of a tissue homogenizer. During the procedure, samples were kept on ice. Samples were centrifuged (1min at 300G, 4°C), and the supernatant was used as whole muscle homogenate and stored at -80°C.

SDS-Page and western blot myosin-actin ratio

To investigate if selective myosin loss is found in this model, we assessed the myosin-actin ratio in muscle tissue of E+A as compared to C+A. Total protein content of whole muscle homogenate was determined using the Lowry protein assay.[16] Samples were normalized to an equal total protein concentration. Equal volumes were mixed with loading buffer (SDS, bromophenol blue, Tris Base, glycerol, mercaptoethanol, and milliQ water) and boiled at 95 °C for 5 minutes. After short centrifugation, equal amounts (10μg protein) were loaded on Criterion™ XT precast gels (Bio-Rad). After sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), gel content was transferred to Immobilin-FL membranes (Merck Millipore, Billerica, USA) by tank blotting. Membranes were blocked for 1 hour in Odyssey blocking buffer (LI-COR, Westburg, Leusden, The Netherlands) at room temperature. Thereafter, membranes were incubated overnight at 4°C in Odyssey blocking buffer containing the primary antibodies anti-myosin (MF-20, DSHB, Iowa City, IA, USA, 1:20000) and anti-actin (Sigma-Aldrich, Zwijndrecht, the Netherlands 1:500) and 0.1% Tween-20. After washing with ice cold TBS-T, membranes were incubated with IRDye 800CW goat anti-rabbit (green) and 680CW goat anti-mouse (red) antibodies in Odyssey blocking buffer with 0.1%Tween-20 for 1 hour at room temperature. After washing with TBS-T, 2-color fluorescent bands were visualized by a 2-channel laser system, bands were identified by their molecular weight (myosin 200kDA, actin 42kDa), and were quantified (Odyssey IR Imager®; LI-COR Biosciences, Bad Homburg, Germany). Equal loading of the protein to the gel was ensured by Coomassie blue staining.

Power calculation and statistical analysis

A power calculation was not performed for this study, because no data on grip strength in this animal model were available to support a power calculation.

Depending on the distribution of the data, means with standard deviation (±SD), medians with interquartile range (IQR), or range and proportions with percentages and total numbers are presented. The Welch's *t*-test was used for assessment of differences between normally distributed variables, and Wilcoxon rank-sum test was used for differences between nonnormally distributed continuous variables.

Differences in grip strength over time between the groups were studied with linear mixed-effects models to account for repeated measures. Separate models for young and old mice were made. As fixed effects, *E. coli* (no/yes) and antibiotics (no/yes) were entered into the model. A mouse identification number was included as a random effect. Round (1/2) added as a fixed effect did not improve model fit and was therefore not included in the final model.

We did 2 subgroup analyses of more severely ill mice: a) mice with bacterial outgrowth in blood and b) mice with severe weight loss (≥10% maximal weight loss).

Statistical significance was defined as *P* < 0.05. Analyses were done using R (version: 3.02; R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Body weight and bacterial outgrowth

In young mice, the maximum weight loss in E+A mice compared to baseline body weight was 10.4% (median, IQR 9.7 - 11.7) vs 2.5% in C+A mice (median, IQR 1.9 - 2.9) and 2.1% in C mice (median, IQR 1.5- 3.4)(*P*<0.001). In old mice, the maximum weight loss in E+A mice was 7.2% (median, IQR 5.3 - 8.5) vs 1.0% in C+A mice (median, IQR 0.0 - 1.4) and 0% in C mice (median, IQR -0.7-1.1)(*P*<0.001).

One mouse in the E+A group died due to severe illness before the end of the experiment.

In the 30 young mice of the E+A group, 22 (73%) showed bacterial outgrowth in spleen, 8 (27%) in liver, and none in blood. In 29 old mice, 25 (86%) showed bacterial outgrowth in spleen, 13 (45%) in liver, and 8 (28%) in blood.

Grip strength

At baseline, mean normalized grip strength was lower in old mice (3.3 g/g body weight, SD 0.4) compared to young mice (4.4 g/g body weight, SD 0.5; *P*<0.01) (Figure 1). A decrease in grip strength over time was seen in all groups. *E. coli* infection was not associated with a decrease in grip strength (Table 2). In the model for young mice, antibiotic administration was associated with a minor increase in grip strength (Table 2).
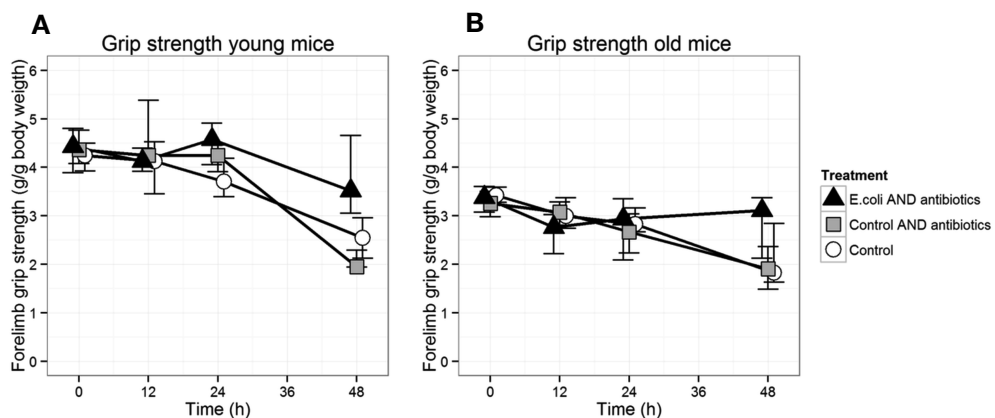
Figure 1. Grip strength in young and old mice
Forelimb grip strength normalized for body weight in young (panel A) and old (panel B) mice at different time points (presented as median and interquartile range).

Subgroup analysis of 8 old mice with bacterial outgrowth in blood showed similar results compared to the whole group analysis of old mice. Also, subgroup analysis of animals with a maximum weight loss ≥ 10% (16 young and 5 old mice) showed similar results.

Table 2. Mixed-effects models for the effect of *E. coli* and antibiotics on normalized grip strength.

	Predicted effect on grip strength (g/g)	95% confidence interval	*P*-value
Model young mice			
E. coli	-0.13	-0.44 - 0.19	0.42
Antibiotics	0.42	0.04 - 0.79	0.03
Model old mice			
E. coli	-0.06	-0.32 - 0.20	0.64
Antibiotics	-0.12	-0.43 - 0.19	0.44

Western blot myosin-actin ratio

In young mice no differences in the myosin-actin ratio were seen between E+A and C+A groups (Figures 2 and 3). In old mice sacrificed at 48 hours, the myosin-actin ratio was lower in the E+A group compared to C+A groups (P=0.01). An opposite effect was found in old mice sacrificed at 72 hours with a higher myosin-actin ratio in the E+A group compared to C+A groups (P=0.01).
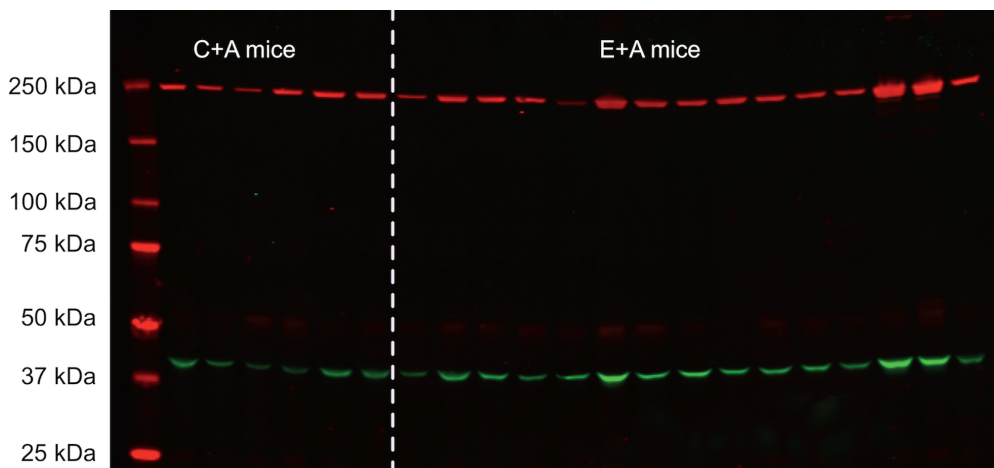
Figure 2. Western blot of myosin (red bands) and actin (green bands).
C+A=control and antibiotics; E+A=*E. coli* and antibiotics.
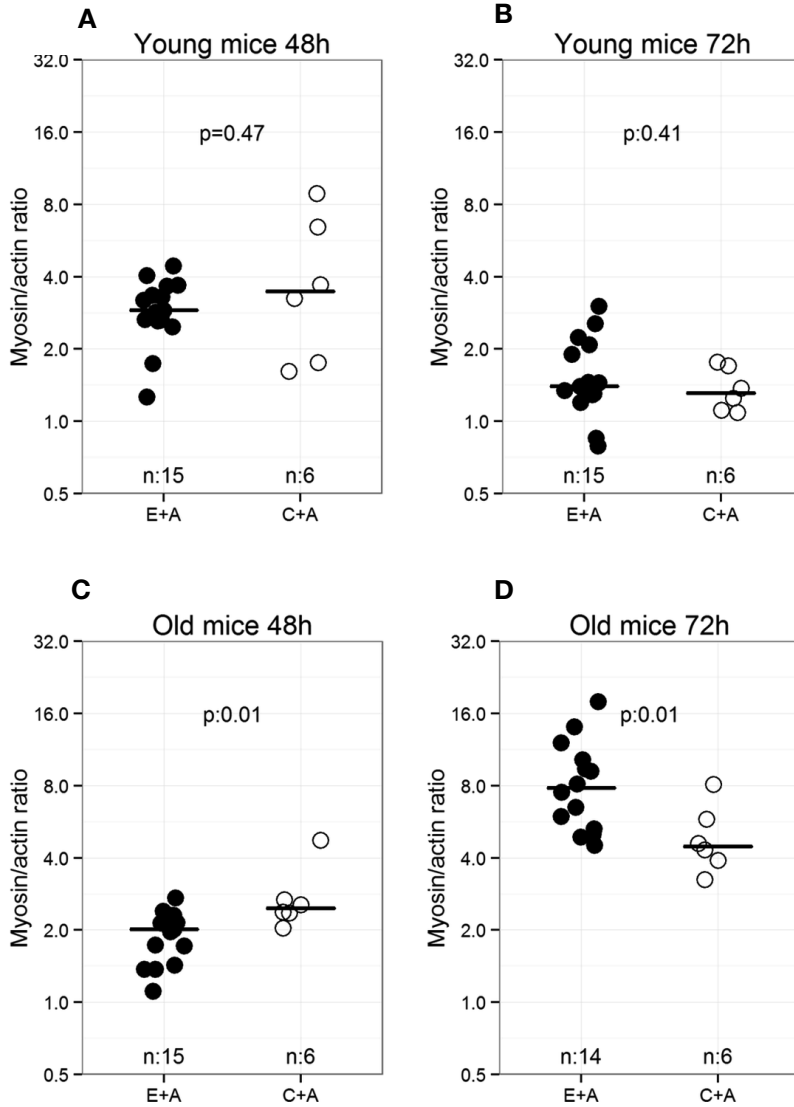
Figure 3. Myosin-actin ratio
Quantified myosin-actin ratio in young (panels A and B) and old mice (panels C and D) at 48 and 72 hours. Horizontal bars represent median values.
E+A=*E. coli* and antibiotics; C+A=control and antibiotics.

DISCUSSION

In this *E. coli* septic peritonitis model, there was no difference in grip strength decline between mice with *E. coli* infection and the control groups, in young as well as old mice.

Both infection and control groups showed a decline in grip strength between 24 and 48 hours. This might be explained by learning bias, since frequent testing has been reported to lead to a loss of interest in holding the grip.[17,18] The unwillingness of the mice also requires multiple trials per animal per time point to generate reliable data.[17]

Only 2 other studies used *in vivo* strength measurements in awake animals to investigate an ICU-AW model.[19,20] Files et al, used the same test as we used and investigated grip strength in mice after intratracheal lipopolysaccharide (LPS) administration.[19] They found a decline in grip strength within 2 days, which remained stable thereafter until 9 days. However, the sham group also showed a temporary decline. Another method of strength assessment was used in a cecal ligation and puncture (CLP) model in rats. Half of the rats in the CLP group were unable to stand on a rotated screen at 7, 14, and 21 days after CLP, while sham rats did not show weakness.[20] The use of CLP as a sepsis model is under debate; low consistency is a major problem, because outcome after CLP is associated with the technical procedures.[21]

Age dependent grip strength

In healthy human and mouse models, grip strength decreases with increasing age.[22,23] This is the first animal study to assess age-dependent grip strength in a sepsis model. Since grip strength is used as a marker of frailty, and age is a risk factor for developing ICU-AW, one would expect that older mice are more susceptible to a decline in grip strength than younger mice.[24] In our model, grip strength at baseline was lower in old mice, as expected, however the decline in grip strength did not seem to be different between young and old mice.

Myosin-actin ratio

In patients with ICU-AW, a decreased myosin-actin ratio is seen within 5 days after ICU-admission.[25] In old mice there was a difference in the myosin-action ratio between the C+A and the E+A group. At 48 hours after *E. coli* administration the myosin-actin ratio was lower in the E+A group, whereas it was higher at 72 hours. The low myosin-actin ratio at 48 hours might reflect a subclinical ICU-AW, since grip strength was not lower in the E+A group. This is an important finding pointing to the need to interpret *in vitro* findings together with functional measurements in order to ascertain their relevance. An overshoot of compensatory production of myosin might explain the higher ratio at 72 hours, although in humans persistent myosin loss has been seen until day 15 after ICU admission.[25] We did not assess this further in our model.

Suitability of this model to study ICU-AW

No difference in grip strength decline was found between mice with *E. coli* infection and the control groups, irrespective of age. Therefore, the *E. coli* mouse model, in its current form, seems to be unsuitable to study ICU-AW.

Several factors may be important. First of all, the illness in this model may not be sufficiently severe and prolonged, so that the threshold to develop ICU-AW was not reached in young or old mice. Previous experiments with this model without antibiotic administration showed full-blown sepsis at 15 hours after induction and a high mortality of 75 to 100% within 48 hours.[12] In our experiment, early antibiotic administration may have prevented prolonged illness. Although we found significant weight loss, indicating illness, none of the young and only 28% of old mice showed bacterial outgrowth in blood. Subgroup analyses of mice with bacterial outgrowth in blood and mice with severe weight loss did not show different grip strength results.

Second, grip strength testing may not be the best method to assess *in vivo* muscle strength in awake mice, since frequent measurements seem to cause learning bias. Other methods to assess *in vivo* muscle strength such as rotarod test or inverted screen test might be useful to detect changes in strength over time in awake mice.[17] Finally, the duration of this model might be too short to detect weakness. In other animal models, it took several days before weakness was detectable.[19,20] Even so, learning bias may hamper sequential strength measurements in longer duration models.

Limitations of this study

We did not perform a power calculation, as this was an exploratory study on grip strength, which was never measured in this model before. However, the exploratory nature of this study allowed us to combine animal experiments and contributed to use of fewer animals. This experiment was primarily designed and powered to study microglia activation in sepsis.

Because of combining animal experiments we were not able to perform other *in vivo* strength measurements or electrophysiological studies.

Recommendations for future research

To study ICU-AW, an animal model with a decline in muscle strength, assessed with *in vivo* strength measurements in awake mice during sepsis, is needed, since a decline in muscle strength is the most important feature of ICU-AW in humans. A prolonged model, with prolonged and severe critical illness might be needed to induce *in vivo* ICU-AW in mice.

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CONCLUSION

This *E. coli* septic peritonitis mouse model did not induce decreased grip strength. No effect of age on grip strength decline could be found in this model.

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FINANCIAL DISCLOSURE

CONFLICT OF INTEREST

Prof. I.N. van Schaik received departmental honoraria for serving on scientific advisory boards and a steering committee for CSL-Behring.

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CHAPTER 7

Muscle weakness in a *S. pneumoniae* sepsis mouse model

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Coen A.C. Ottenheijm, Marcus J. Schultz, Ivo N. van Schaik, Janneke Horn

ABSTRACT

Introduction

The pathophysiology of intensive care unit-acquired weakness (ICU-AW), which affects peripheral nerves, limb muscles and respiratory muscles, is complex and incompletely understood. This illustrates the need for an ICU-AW animal model. However, a translatable and easily applicable ICU-AW animal model does not exist. The objective of this study was to investigate whether induction of a *S. pneumoniae* sepsis could serve as a model for ICU-AW.

Methods

A total of 24 C57BL/6J mice were infected intranasally with viable *S. pneumoniae*. Control mice (n=8) received intranasal saline. Ceftriaxone was administered at 24h (n=8) or at 48h after inoculation (n=8), or as soon as mice lost 10% of their body weight (n=8). The primary endpoint, *in vivo* grip strength, was measured daily. At the end of the experiment, at 120h after inoculation, electrophysiological recordings were performed and diaphragm muscle was excised to determine *ex vivo* muscle fiber strength and myosin/action ratio.

Results

Grip strength over time was similar between experimental and control groups and electrophysiological recordings did not show signs of ICU-AW. Diaphragm fiber contractility measurements showed reduced strength in the group that received ceftriaxone at 48h after *S. pneumoniae* inoculation.

Conclusion

Diaphragm weakness, but no limb weakness was found in the *S. pneumoniae* mouse model in which severe illness was induced. This does not reflect the full clinical picture of ICU-AW as seen in humans and as such this model did not fulfill our predefined requirements. However, this model may be used to study inflammation induced diaphragmatic weakness.

INTRODUCTION

Intensive care unit-acquired weakness (ICU-AW) is an important complication of critical illness. It is caused by dysfunction or structural damage of nerves (critical illness polyneuropathy, CIP), muscles (critical illness myopathy, CIM) or both (critical illness neuromyopathy, CINM). ICU-AW is characterized by diffuse weakness of both limb and respiratory muscles.[1,2] Selective myosin filament loss is characteristic of CIM and can be seen within 5 days after ICU admission.[3]

The main risk factors for ICU-AW are sepsis, the systemic inflammatory response syndrome (SIRS) and multiple organ failure.[4] The pathophysiology is complex and not completely understood, which seriously hampers the development of potential therapeutic possibilities for ICU-AW.

To further unravel the pathophysiology of ICU-AW, an animal model is needed. Several animal models have been used to study muscle dysfunction in ICU-AW, but all these models have their limitations. Some frequently used models are far away from the human ICU situation, e.g. the model of muscle denervation and dexamethasone treatment[5,6], or have low consistency and reproducibility, e.g. the cecal ligation and puncture (CLP) model to induce sepsis[7]. Others mimic ICU conditions more closely, but are very expensive and time-consuming because they need continuous monitoring, e.g. porcine[8] or rat[9] models with several days of mechanical ventilation. Another important limitation of the existing models is that *in vivo* strength measurements have been scarcely performed, whereas decreased muscle strength is a prerequisite for the diagnosis of ICU-AW in humans.[10] Only surrogate markers for muscle strength like electrophysiological studies and contractility measurements have been used.[5,8] The translatability of these models to ICU-AW in humans is therefore uncertain.

We aimed to use a well-known and easily applicable *S. pneumoniae* sepsis model to induce ICU-AW in mice.[11,12] The primary objective of this study was to investigate whether this sepsis model could serve as an ICU-AW model that more resembles the human disease, using *in vivo* muscle strength measurements as our primary endpoint.

METHODS

Animals and ethical approval

A total of 36, 8-10 weeks old male, specific-pathogen-free, C57BL/6J mice were obtained from Charles River. To acclimatize, mice were housed in groups of four in individually ventilated cages for nine days before the start of the experiment. Food and water were available ad libitum and a 12:12 hour light-dark cycle was retained.

All experiments conformed the Dutch Experiments on Animals Act for the care and use of animals and were approved by the Institutional Animal Care and Use

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Committee of the Academic Medical Center, Amsterdam, the Netherlands (permit number: 102904).

This manuscript was drafted in accordance with the ARRIVE (Animal Research: Reporting of *In Vivo* Experiments) guidelines.[13]

Induction of pneumonia

Mice were inoculated intranasally with *S. pneumoniae* as described elsewhere [14]. In short, *S. pneumoniae* serotype 3 (American Type Culture Collection 603) were cultured in Todd-Hewitt broth at 37°C in 5% CO_2 for 4h to a mid-logarithmic phase, harvested by centrifugation at 4000 rpm for 10 min, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline and diluted to a concentration of ~4 X10^6 colony-forming units (CFU)/ml, as determined by plating serial 10-fold dilutions onto sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane and 50 µl (~2x10^5 CFU) was inoculated intranasally.
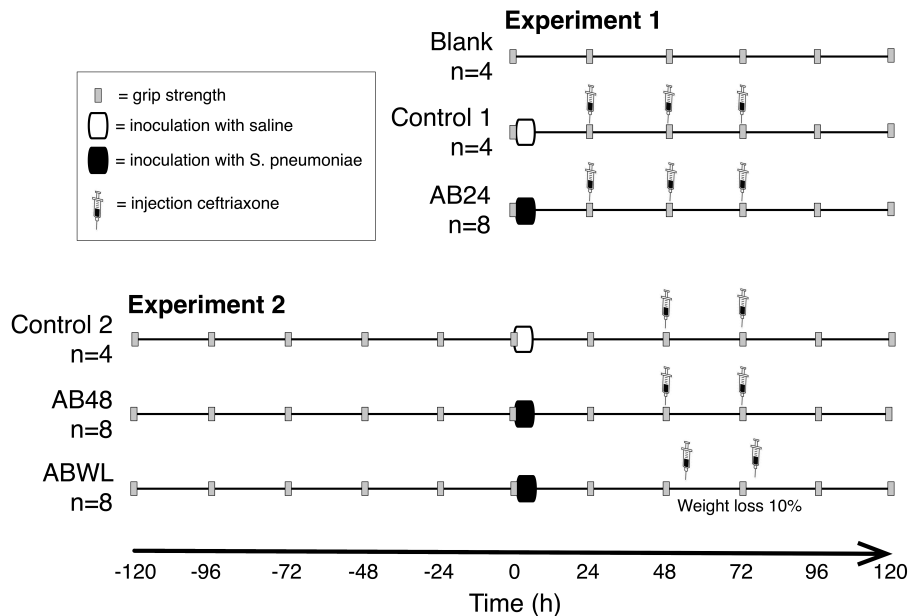
Figure 1. Experimental groups
Overview of experimental procedures. Grip strength was measured daily and started at the time of inoculation in experiment 1 and five days before inoculation in experiment 2. Mice of the Blank group did not receive any inoculation nor any intraperitoneal injections with ceftriaxone (antibiotics). Mice of the control groups were inoculated with saline and the other experimental groups with ~2x10^5 colony forming units of *S. pneumoniae*. Mice of the control 1 and AB24 group received their first ceftriaxone administration at 24 hours after inoculation, mice of the control 2 and AB48 group at 48 hours after inoculation and mice of the ABWL group as soon as they lost 10% of their body weight. Mice in experiment 1 received a total of three injections with ceftriaxone and mice in experiment 2 received two injections.

Experimental procedures

Experimental procedures of experiment 1 and 2 are presented in figure 1. In experiment 1, mice were assigned to three groups (random per cage): Blank group (n=4), Control1 group (n=4) and *S. pneumoniae* and antibiotics group (AB24, n=8). Mice of the Blank group did not receive any inoculation under isoflurane nor any intraperitoneal injections. At the start of the experiment (t=0), mice of the AB24 group were inoculated with *S. pneumoniae*, while the Control1 group received 50μl of sterile isotonic saline intranasally. To mimic human sepsis treatment, improve survival and maintain fluid volume status, mice received intraperitoneal injections with antibiotics in saline. Mice of the AB24 and Control1 group received ceftriaxone (Fresenius Kabi, Den Bosch, the Netherlands), 20mg/kg and 10μl/g body weight at 24, 48 and 72 hours after inoculation. Body weight and discomfort, such as reduced locomotor activity, were measured at least twice daily.

In experiment 2, mice were assigned to three groups (random per cage): Control2 group (n=4), *S. pneumoniae* and antibiotics group (AB48, n=8) and *S. pneumoniae* and antibiotics at weight loss group (ABWL, n=8). The first antibiotic treatment was either delayed for another 24 hours or administered as soon as mice lost 10% of their body weight (compared to the maximal weight until that time point). Also, mice received two instead of three recurrent injections of ceftriaxone (20mg/kg and 10μl/g body weight). Inoculation with *S. pneumoniae* or saline was performed as in experiment 1. At 48 and 72 hours after inoculation, mice of the Control2 and AB48 group received an intraperitoneal injection with ceftriaxone. Mice of the ABWL group received ceftriaxone as soon as they lost 10% of their body and 24 hours thereafter. Body weight and discomfort was measured at least three times daily.

Grip strength testing

Fore limb grip strength was measured daily (~9.00 AM) by an experienced investigator (EW) using a grip strength meter with metal grid (Bioseb, France) as previously described.[15] At each time point, three grip strength measurements were taken, of which the average result was used for analysis. Grip strength was normalized for concomitant body weight.[16]

In experiment 2, grip strength measurements were taken daily from 120 hours before to 120 hours after inoculation.

Electrophysiological recordings

At the end of the experiment, at t=120 hours after inoculation, mice were anesthetized by inhalation of isoflurane. Tail and hind limbs were strapped to a board and a heating pad maintained body temperature. The sciatic and caudal nerves were studied on one side on a Viking III EMG machine (Nicolet, Madison, USA) by insertion of stimulating and recording monopolar needle electrodes

followed by supramaximal stimulation. Compound muscle action potentials (CMAPs) amplitudes (peak to peak) of the sciatic nerve were recorded and motor nerve conduction velocities (NCVs) over the segment between the ankle and the sciatic notch were calculated. For studies of the caudal nerve, compound nerve action potentials (CNAPs) amplitudes (baseline to negative peak) were recorded and NCVs were calculated.

Euthanasia

After the electrophysiological recordings, mice were euthanized by an intraperitoneal injection of a mix of 126 mg/kg ketamine (Nimatek, Eurovet Animal Health BV, The Netherlands), 0.1mg/kg dexmedetomidine (Dexdomitor, Orion pharma, Finland), and 0.5 mg/kg atropine (Pharmachemie BV, The Netherlands) in sterile saline followed by dissection of the carotid artery.

Lung and spleen were harvested for determination of bacterial outgrowth. Fresh muscle strips, dissected from the excised diaphragm, were placed overnight at 5°C in relaxing solution (for composition, see below) containing 1% Triton X-100 to permeabilize the membranes. Subsequently, the specimens were washed overnight with relaxing solution, then placed in a 50% glycerol/relaxing solution (vol/vol), and stored at -20°C until further use.

Bacterial outgrowth

Lung and spleen were homogenized in four volumes of sterile saline with a tissue homogenizer. Serial ten-fold dilutions of the homogenates were plated on sheep-blood agar plates and bacteria were allowed to grow at 37°C.

Diaphragm fiber contractility measurements and myosin-actin ratio measurements

As it is not possible to measure diaphragm strength in awake mice and in vivo electrophysiological recordings of diaphragm in mice are hard to perform, we investigated diaphragm strength ex vivo in the second experiment. Fiber contractile measurements and experimental protocols were performed according to previously described methods with minor modifications.[17]

Small bundles of fibers (100-150 um in diameter) were isolated from the diaphragm samples using micro forceps. The fiber ends were attached to aluminum-foil clips and mounted on a muscle-fiber apparatus (Aurora Scientific, Aurora, Ontario, Canada), which was placed on top of an inverted microscope. One end of the fiber bundle was attached to a force transducer (model 403A, Aurora Scientific), whereas the other end was attached to a servomotor (315C, Aurora Scientific). Preparations that appeared damaged during microscopic examination were

excluded from the study. The number of excluded fiber bundles did not differ per group. All measurements were performed at 20°C.

The composition of relaxing solution (total ionic strength of 180mM) consisted of 5.89mM Na_2ATP, 6.48mM $MgCl_2$, 40.76mM K-propionate, 100mM BES, 6.97mM EGTA and 14.5mM CrP with sufficient KOH to adjust the pH to 7.1. The negative logarithm of the free Ca^{2+} concentration (pCa) of the relaxing solution was set 9.0, whereas the activating solution was set at pCa 4.5.

While in relaxing solution, sarcomere length was set at 2.5 µm using a fast Fourier transformation on a region of interest on the real time camera image. Muscle fiber length and thickness (in both x-y and x-z direction) were measured using the live camera image. The cross sectional area (CSA) was calculated from the thickness measurements, assuming that the cross section of the fiber bundle is ellipsoidal. All active forces were measured at a sarcomere length of 2.5µm and are expressed as tension (force per CSA).

The fiber bundle was placed for 1 minute in pre-activating solution before maximal isometric force was measured upon placement of the fiber in activating solution (pCa 4.5). The rate constant of force redevelopment (k_{tr}) was measured during maximal activation (pCa 4.5) by rapidly releasing the fiber by 30% of its original length, followed by a quick restretch to its original length. This release detaches all myosin heads attached to actin, and subsequently force redevelops. The k_{tr} was determined by fitting a double exponential through the force redevelopment curve (note that only the fast rate constant is reported as this is considered to reflect cross-bridge cycling kinetics). Active stiffness was determined during maximal activation (pCa 4.5) by imposing small length perturbations (0.3, 0.6, 0.9% of initial length) on the fiber bundle resulting in a quick force response. The tension change (ΔT) was plotted as a function of the length change (ΔL). Active stiffness was derived from the slope of the fitted line and is a measure to estimate the number of cycling cross-bridges. The ratio of maximal tension and active stiffness reflects the force generated per cross-bridge.[18]

Finally, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to determine the myosin-actin ratio in the muscle fiber preparations that we used in our contractility experiments. Muscles fibers were de-natured by boiling at 80°C for 2 min in SDS sample buffer. Samples were loaded on a 7-15% acrylamide gel. The gels were run for 3 h at 15°C and a constant voltage of 275 V. Finally, the gels were stained with sypro-Ruby, scanned, and the myosin heavy chain and actin protein bands were analyzed with AIDA Image Analyzer software (Raytest Isotopenmessgeräte GmbH, Straubenhardt, Gemany).

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Experimental outcomes

The primary outcome was a decrease in grip strength, and secondary outcomes were differences between experimental and control groups in electrophysiological recordings, diaphragm muscle fiber strength and myosin-actin ratio of the diaphragm.

Power calculation and statistical analysis

We chose the number of animals per group on pragmatic grounds, because no previous data on grip strength in this animal model were available to support a power calculation.

Unless otherwise stated, continuous variables are presented as medians with interquartile range (IQR). Kruskal-Wallis test was used to compare continuous variables between the three groups in each experiment. The experimental groups were compared to the control groups by use of the Mann-Whitney U test. Weight loss was calculated by the percentage of difference between the weight and the maximal weight until that time point.

For the analysis of diaphragm muscle contractility normal distribution was tested. If data was normally distributed multilevel analysis to correct for non-independence of successive measurements per animal (MLwiN, 2.02.3; Center for Multilevel Modelling, Bristol, UK) was used.[17,18]

Statistical significance was defined as $p < 0.05$. Analyses were done using R (version: 3.02; R Foundation for Statistical Computing, Vienna, Austria).

Table 1 Mouse characteristics

Characteristic	Experiment 1				Experiment 2			
Group, number	**Blank N=4**	**Control1 N=4**	**AB24 N=8**	**P-value**	**Control2 N=4**	**AB48 N=8**	**ABWL N=8**	**P-value**
Body length[§], mm	90.0 [90.0-92.0]	90.0 [89.5-91.0]	90 [89.8-90.8]	0.70	92.0 [89.5-93.5]	92.5 [91.3-93.8]	91.5 [90.3-93.5]	0.80
Baseline weight t=0, gr	25.1 [24.8-26.4]	24.8 [24.0-25.4]	25.4 [24.6-25.8]	0.69	27.4 [27.0-27.8]	26.4 [25.2-28.4]	27.0 [25.9-27.8]	0.72
Maximal weight loss, %	4.5 [4.2-4.8]	3.8 [3.4-4.5]	5.0 [3.7-7.3]	0.46	4.1 [3.5-4.6]	15.2 [11.6-17.0]*	14.2 [12.8-15.5]*	0.01

[§]nose to base of tail, *p<0.05 compared to Control2 group.
AB24=first antibiotics administered at 24 hours after inoculation with *S. pneumonia*; AB48=first antibiotics administered at 48 hours after inoculation with *S. pneumonia*; ABWL=first antibiotics administered as soon as 10% of body weight was lost after inoculation with *S. pneumoniae.*

A   Weight loss experiment 1

B   Weight loss experiment 2
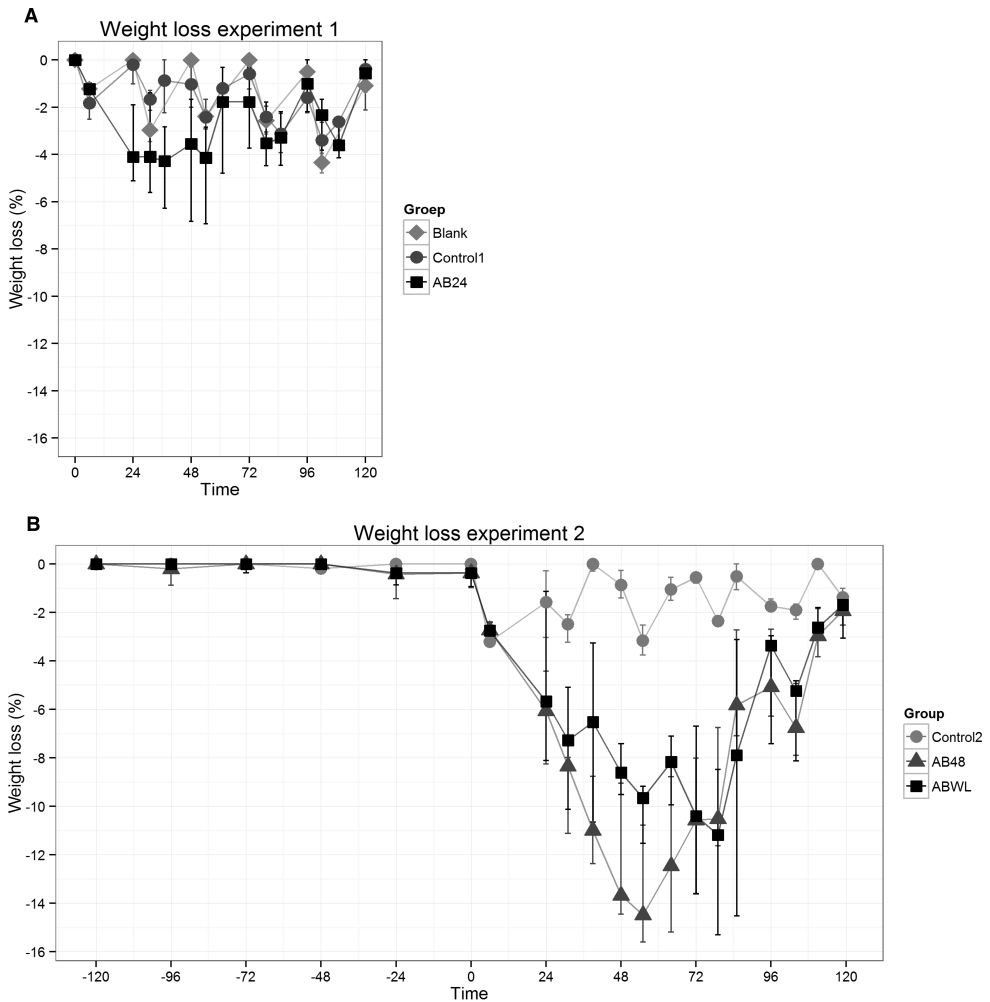
Figure 2. Weight loss over time
Weight loss over time in experiment 1 (panel A) and 2 (panel B) compared to maximal weight until that time. Mice in experiment 1 showed no significant weight loss compared to the control groups. Mice in experiment 2 (AB48 and ABWL groups) showed severe weight loss compared to the control group. Percentage weight loss is presented as median and interquartile range.

RESULTS

Baseline bodyweight and length were not significantly different between the various experimental groups for both experiments (table 1).

Clinical illness severity and bacterial outgrowth

Mice of the AB24 group of experiment 1 showed slightly reduced locomotor activity. Maximal weight loss during the experiment, as a measure of illness severity, was 5.0% (median, IQR 3.7-7.3) in the AB24 group and was equal to the Control1 and Blank group (p=0.46; table 1 and figure 2A).

Because mice did not develop severe illness in experiment 1, which was possibly prevented by early and recurrent administration of antibiotics, in experiment 2 the first antibiotic treatment was either delayed for another 24 hours or administered as soon as mice lost 10% of their body weight. Furthermore, antibiotics were administered twice instead of three times. As a result, mice in the AB48 and ABWL group became more severely ill, represented by considerable reduced locomotor activity and a significant weight loss of 15.2% (IQR 11.6-17.0) in the AB48 group and 14.2% (IQR 12.8-15.5) in the ABWL group (p<0.05 compared to the Control2 group (4.1% (IQR 3.5-4.6); table 1, figure 2B). In the ABWL group the first administration of antibiotics was at 47 hours (median, IQR 31-68).

Two mice (one in the AB48 and one in the ABWL group) died before the end of the experiment and two other mice (one in the AB48 and one in the ABWL group) had a hunched posture and severely decreased locomotor activity and had to be euthanized before the end of the experiment according to predefined humane endpoints.

In experiment 1, homogenates of lung and spleen showed no bacterial outgrowth. In experiment 2, five of six lung homogenates of the AB48 group and all six spleen homogenates showed bacterial outgrowth. In the ABWL group all of six lung and three of six spleen homogenates showed bacterial outgrowth.

Grip strength

Results of grip strength measurements over time are presented in figure 3. In experiment 1, grip strength declined in all groups, with the biggest drop within the first 72 hours after inoculation. No differences were seen between the experimental and control groups. Also, in the Blank group, the group that did not receive any interventions such as inoculation or injections, grip strength declined. Because of this decline in grip strength in the first few days in all groups of experiment 1, we chose to incorporate a grip strength training phase in experiment 2, starting five days before inoculation. Despite the fact that mice in the AB48 and ABWL group became more severely ill, no differences were seen between the experimental and control groups during 5 days of follow-up.

A

B
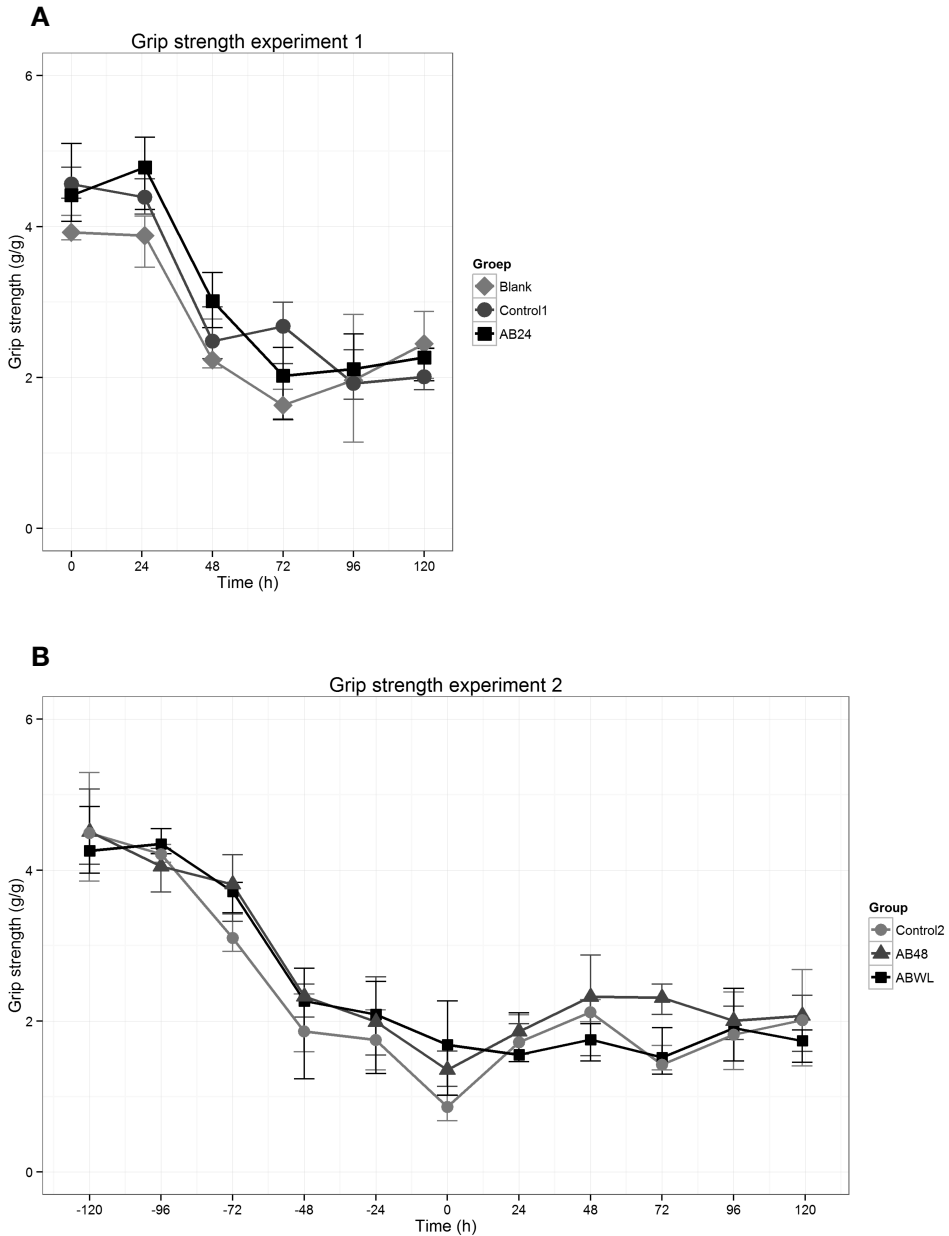
Figure 3. Corrected mean grip strength
Fore limb corrected mean grip strength (median and interquartile range) over time in experiment 1 (panel A) and experiment 2 (panel B). Experiment 2 includes a grip strength training phase (from -120 h to 0 h). Grip strength declined in the first 3-4 days in all groups. No differences in grip strength decline were seen between the groups.

Electrophysiological recordings

Results of electrophysiological recordings are presented in table 2. No differences in electrophysiological recordings were found between the groups in experiment 1. In experiment 2, CMAPs of the sciatic nerve were higher in the ABWL group compared to the Control2 group. NCV of the caudal nerve was higher in the AB48 group compared to the Control2 group.

Table 2. Electrophysiological measurements of the sciatic and caudal nerve

	Experiment 1				Experiment 2			
	Blank N=4	Control1 N=4	AB24 N=8	P-value	Control2 N=4	AB48 N=6	ABWL N=6	P-value
Sciatic nerve								
CMAP(mV)	7.9 [5.1-11.4]	8.7 [6.7-10.0]	11.7 [9.2-13.9]	0.43	5.3 [3.1-7.8]	8.9 [7.7-11.0]	11.8 [9.2-15.2]*	0.08
NCV (m/s)	43.0 [41.8-47.5]	42.5 [41.8-43.5]	42.5 [42.0-45.0]	0.91	37 [34.5-41.0]	36 [31.5-39.8]	31 [30.0-33.0]	0.21
Caudal nerve								
CNAP (µV)	114.8 [101.7-140.9]	110.3 [93.9-128.3]	131.1 [118.2-146.0]	0.50	106.4 [100.0-122.5]	111.8 [95.3-124.4]	129.3 [98.1-145.7]	0.94
NCV (m/s)	41.0 [38.8-43.3]	40.0 [38.8-41.3]	41.0 [38.5-42.5]	0.85	33.5 [29.8-37.3]	44.5 [41.0-47.3]*	41.0 [38.8-42.5]	0.05

*p<0.05 compared to Control2 group. Nerve conduction measurements presented as median with interquartile range.
CMAP=compound muscle action potential; CNAP=compound nerve action potential; NCV=nerve conduction velocity.

Muscle fiber contractility

Muscle fiber contractility was measured in diaphragm samples of nine mice, three of each group of experiment 2. Maximal tension (force per CSA) was lower in the AB48 group compared to the control2 group (41.1 nM/mm^2 versus 65.8 nM/mm^2, p=0.0084) (figure 4A). A reduction in tension in single muscle fibers can be explained by one or more of the following factors: the number of available cross bridges, the fraction of strongly bound cross-bridges and the force per cross-bridge.[19] There were no changes in the rate constant of force redevelopment (k_{tr}) indicating that the fraction of strongly bound cross bridges was unaltered (figure 4B). The force

generated per cross bridge was also not different (i.e. the ratio of maximal tension and active stiffness) (figure 4D). This indicates that the lower maximal tension in the AB48 group is caused by a limited number of available cross bridges.
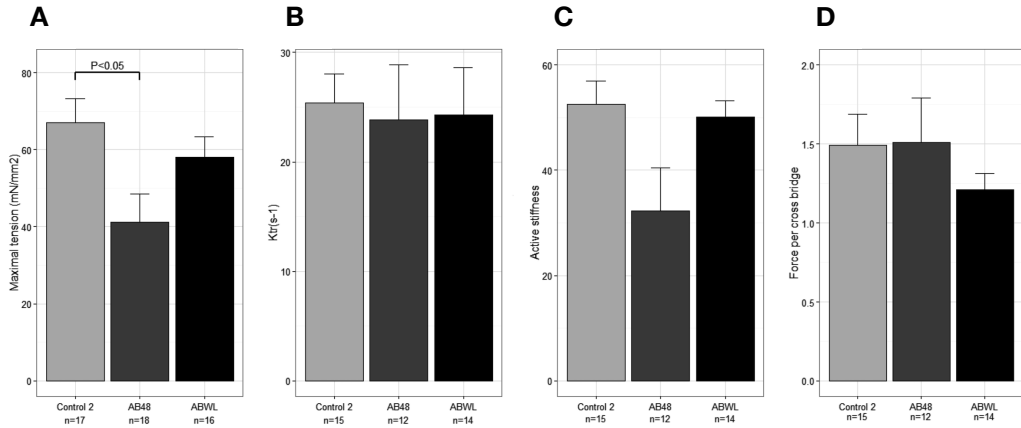
Figure 4. Diaphragm fiber contractility
Contractility measurements of individual diaphragm fibers. (A) Maximal tension, which is the absolute maximal force normalized to fiber cross-sectional area, was significantly lower in fibers of the AB48 group compared to control fibers. (B) There were no changes in the rate constant of force redevelopment (k_{tr}) indicating that the fraction of strongly bound cross bridges was unaltered. (C) Active stiffness was not statistically different between the groups. (D) The force generated per cross bridge (i.e. the ratio of maximal tension and active stiffness) was not different between the groups.
Values are presented as mean and error bars represent standard error of the mean. In each group diaphragm fibers of three mice were assessed. Numbers below the graphs are total numbers of fibers analyzed.

Myosin-actin ratio in diaphragm

Myosin-actin ratio was determined in all diaphragm fiber bundle of the contractility measurements. There were no differences in myosin-actin ratio between the groups (Figure 5).
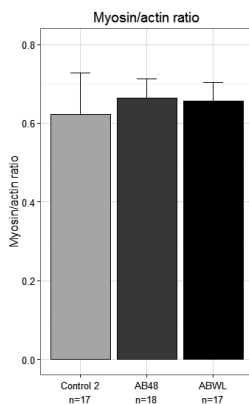
Figure 5. Myosin-actin ratios
Myosin-actin ratio of diaphragm fibers determined by western blot showed no differences between the groups. Values are presented as mean and error bars represent standard error of the mean. In each group diaphragm fibers of three mice were assessed. Numbers below the graphs are total numbers of fibers analyzed.

DISCUSSION

Grip strength and electrophysiological measurements

In our *S. pneumoniae* model experiments, grip strength decreased, but there was no difference between control and experimental groups. In the first experiment we found a decline in grip strength within the first 72 hours in all groups, including the mice that did not underwent any intervention. We also saw this decline in a previous experiment with an *E. coli* infection.[15] In fact, a risk for learning bias has been described with frequent grip strength testing.[20] Therefore, we included a training phase in our subsequent experiment. We started grip strength testing five days before inoculation in an attempt to diminish this effect. In this experiment again a decline in grip strength was seen during the training phase. After inoculation strength remained stable without any differences between the groups. Because of this probable learning effect grip strength testing may not be the best in vivo method to detect acute muscle weakness in awake mice. Other in vivo methods could be considered, such as the rotarod test or inverted screen, although learning bias can also be a problem with the rotarod test.[21]

Besides grip strength we also investigated peripheral nerve or muscle involvement by electrophysiological recordings. The most common findings in nerve conduction studies in ICU-AW patients are reduced CMAP amplitudes (in CIM and CIP) and/or SNAP amplitudes (in CIP) and normal or slowed conduction velocities.[1,22] Nerve conduction studies in our experiments did not show abnormalities as can be seen in ICU-AW patients. CMAP amplitudes of the sciatic nerve in the second experiment were even higher in the *S. pneumoniae* groups compared to the control group. We do regard this as a co-incidence, which may be due to the small sample size. Because of their possible harm, electrophysiological measurements were only done at the end of the experiment. Reversible changes may thus have been missed. We did not perform needle electromyography to detect abnormal spontaneous activity, which may be more sensitive than nerve conduction studies to detect muscle damage, either primary or secondary to nerve damage.[23]

Diaphragm contractility and myosin-actin ratio

Because the diaphragm is also effected by ICU-AW and diaphragm weakness is a major cause for prolonged mechanical ventilation, we also investigated the diaphragm from severely ill mice.[24,25] Besides mechanical ventilation[26], inflammation[27] also causes diaphragmatic weakness.

Muscle fiber contractility measurements of the diaphragm showed a reduction of maximal tension in mice of the AB48 group compared to controls. This was explained by a limited number of available cross bridges. However, myosin-actin ratio was not different between these groups. This suggests that, instead of

selective myosin loss, which is described in ICU-AW, part of the contractile material is damaged and is therefore not functional anymore. This is in line with findings from diaphragm fibers of critically ill patients who were mechanically ventilated for a median 88 hours prior to biopsy.[28] Individual diaphragm fibers of these patients were severely weakened, both by atrophy and by dysfunction of the remaining contractile proteins, without evidence for selective myosin loss.

Suitability of this model to study ICU-AW

This model does not capture the full clinical spectrum of ICU-AW as seen in patients. We found no evidence of limb muscle weakness. On the other hand, diaphragmatic weakness was found.

Limb muscle function was assessed with grip strength and nerve conduction studies. As these assessments did not show abnormalities, this model does not resemble patients with ICU-AW. However, we cannot rule out that more sensitive methods such as needle electromyography, and muscle and nerve biopsies, may reveal evidence for limb muscle and peripheral nerve involvement.

Although mice were severely ill and several of the animals died during the experiment, the threshold needed to develop limb weakness may not have been reached in this model. Mice recovered quickly as was also indicated by the restored body weight. In our second experiment, maximum weight loss was reached after 2-3 days and at the end of the experiment body weight was almost on baseline level. However, since 25% of mice in the experimental groups died or had to be euthanized due to too severe illness, further postponing or reducing the antibiotic treatment does not seem to be an option. Pathogens with a more prolonged/chronic critical illness may be used to induce sepsis, or mechanical ventilation could be added to the model to introduce a second hit.

Contractility measurements of the diaphragm showed weakness in the group of mice with the most severe weight loss. Previous animal studies have shown that the diaphragm is more susceptible to systemic inflammation; i.e. diaphragm muscle contractility was decreased, while limb muscle contractility was preserved.[29,30] This *S. pneumoniae* model may be used to further study inflammation induced diaphragm weakness.

Limitations of this study

In addition to the limitations already stated above, this study has some other limitations. We did not perform a power calculation for this study, because no previous data on grip strength in this animal model were available. Secondly, we used different methods to detect weakness in diaphragm and limb muscles. We performed ex vivo contractility measurements of the diaphragm, but we did not perform these measurements in limb muscle. We can therefore not rule out that

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contractility measurements may have shown changes as well in limb muscles, despite a lack of change in grip strength and electrophysiological measurements.

CONCLUSION

Although mice became severely ill, this *S. pneumoniae* mouse model showed no weakness with *in vivo* strength measurements and electrophysiological studies as seen in humans with ICU-AW. As such, this model did not fulfill our predefined requirements. However, weakness of the diaphragm was found, and as weaning from a ventilator is a major issue in human ICU-AW this model may be used to study inflammation induced diaphragmatic weakness.

ACKNOWLEDGEMENTS

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PART III

EARLY DIAGNOSIS AND INDIVIDUAL

PREDICTION OF ICU-AW

CHAPTER 8

Diagnostic accuracy of quantitative neuromuscular ultrasound for the diagnosis of intensive care unit-acquired weakness: a cross-sectional observational study

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ABSTRACT

Background

Neuromuscular ultrasound is a noninvasive investigation, which can be easily performed at the bedside on the ICU. A reduction in muscle thickness and increase in echo intensity over time have been described in ICU patients, but the relation to ICU-acquired weakness (ICU-AW) is unknown. We hypothesized that quantitative assessment of muscle and nerve parameters with ultrasound can differentiate between patients with and without ICU-AW. The aim of this cross-sectional study was to investigate the diagnostic accuracy of neuromuscular ultrasound for diagnosing ICU-AW.

Methods

Newly admitted ICU patients, mechanically ventilated for at least 48 hours, were included. As soon as patients were awake and attentive, an ultrasound was made of four muscles and two nerves (index test) and ICU-AW was evaluated using muscle strength testing (reference standard; ICU-AW defined as mean Medical Research Council score< 4). Diagnostic accuracy of muscle thickness, echo intensity and homogeneity (echo intensity standard deviation) as well as nerve cross-sectional area, thickness, and vascularization were evaluated with the area under the curve of the receiver operating characteristic curve (ROC-AUC). We also evaluated diagnostic accuracy of z-scores of muscle thickness, echo intensity and echo intensity standard deviation.

Results

Seventy-one patients were evaluated of whom 41 had ICU-AW. Ultrasound was done at a median of 7 days after admission in patients without ICU-AW and 9 days in patients with ICU-AW. Diagnostic accuracy of all muscle and nerve parameters was low. ROC-AUC ranged from 51.3 to 68.0% for muscle parameters and from 51.0 to 66.7% for nerve parameters.

Conclusions

Neuromuscular ultrasound does not discriminate between patients with and without ICU-AW at the time the patient awakens, and is therefore not able to reliably diagnose ICU-AW in ICU patients relatively early in the disease course.

BACKGROUND

Intensive care unit-acquired weakness (ICU-AW) is an important cause of morbidity in critically ill patients and develops in approximately 50% of patients in the ICU. [1,2] Patients with ICU-AW suffer from severe weakness affecting all extremities and often fail to wean from the ventilator.[3] ICU-AW is caused by muscle dysfunction (critical illness myopathy; CIM), nerve dysfunction (critical illness polyneuropathy; CIP) or a mixed dysfunction (critical illness neuromyopathy (CINM).[3]

ICU-AW is diagnosed by assessment of manual muscle strength, using the Medical Research Council (MRC) score.[3,4] A major limitation of muscle strength testing is that patients need to be awake and cooperative for reliable assessment.[5] Since consciousness and cooperativeness are often impaired in ICU patients, especially in the first days after ICU admission, a diagnosis of ICU-AW is often delayed.[6] To diagnose ICU-AW at an early stage, other diagnostic methods are needed.

Neuromuscular ultrasound (NMUS) is an upcoming technique to diagnose muscle disorders[7] and peripheral neuropathies[8]. NMUS can detect muscle atrophy and changes in muscle architecture. Muscle echo intensity may increase due to an increase in fat and fibrous tissue.[7] It can be quantified with computer software by calculating the average gray scale level of the muscle, which is more accurate and objective than visual evaluation.[9] Nerve cross-sectional area (CSA) and echo intensity can also be quantified, as well as increased intraneural vascularization.[10]

A limited number of muscle ultrasound studies have been performed in ICU patients, which were recently summarized in two systematic reviews.[11,12] A reduction in muscle thickness[13-20] or CSA[17,21] and increase in echo intensity[16,17,22] over time are reported. As these studies did not discriminate between patients with and patients without ICU-AW, it is unknown whether these changes are specific for ICU-AW and can be used to diagnose ICU-AW, or that these changes are found in all ICU patients. Nerve ultrasound parameters have never been assessed in ICU patients.[11]

The aim of this cross-sectional study was to investigate the diagnostic accuracy of quantitative NMUS for diagnosing ICU-AW. We hypothesized that quantitative NMUS can discriminate between patients with and without ICU-AW at the time the patient awakens.

METHODS

Design and ethical approval

This cross-sectional observational study was performed in the mixed medical-surgical ICU of the Academic Medical Center, Amsterdam, The Netherlands and was designed in accordance with the STARD criteria[23].

The Institutional Review Board of the Academic Medical Center Amsterdam approved the study (NL41156.018.12.; 2012_264 #B2013585a) and the study was registered in the Netherlands Trial Register (NTR4148). Informed consent was obtained from all patients prior to inclusion.

Inclusion and exclusion criteria

Consecutive, newly admitted ICU patients, who were mechanically ventilated for ≥48 hours, were eligible for inclusion. Patients with an admission diagnosis of a neuromuscular disorder, stroke, cardiac arrest, traumatic brain injury, spinal injury, or intracerebral infection or space-occupying lesion, were excluded. In addition, we excluded patients with a poor pre-hospital functional status (modified Rankin >3[24]), preceding spinal injury, and patients in whom no arms or no legs were available for muscle strength testing or ultrasound.

Medical Research Council score (the reference standard)

Muscle strength was assessed as soon as patients were awake (Richmond Agitation Sedation Scale (RASS) between -1 and 1) and cooperative (able to follow 5 verbal commands with facial muscles, as scored by the Score of 5 Questions[24]). Assessment was done by trained and experienced physiotherapists who were blinded for ultrasound results. The MRC score was used for assessment of strength in the following six muscle groups bilaterally: wrist dorsiflexors, elbow flexors, shoulder abductors, hip flexors, knee extensors, and ankle dorsiflexors. ICU-AW was defined as a mean MRC score <4, in accordance with the international consensus statement.[3]

Neuromuscular ultrasound measurements (the index test)

On the same day of the muscle strength assessment, NMUS testing was done by trained assessors (EW or CV) who were blinded for the muscle strength results, using an Esaote MyLabTwice ultrasound machine (Esaote, Genova, Italy). The biceps brachii (BB) muscle, tibialis anterior (TA) muscle and median nerve were measured on the left side of the body, and the rectus femoris (RF) muscle, flexor carpi radialis muscle (FCR) and peroneal nerve on the right side. If ultrasound was not possible on the preferred side, for example due to arterial lines or dressings, the opposite side was studied.

For muscle assessment, a 4-13 MHz linear array transducer was used with constant image acquisition settings, including constant focus. Three independent transverse images were taken per muscle based on predefined anatomical landmarks (figure E1 and table E1 in the data supplement). For assessment of muscle thickness depth settings were adapted, if needed.

For quantitative echo intensity analysis, ultrasound images were analyzed with an in-house developed software routine for MATLAB (R2014b, Mathworks, Natick, MA, USA). Briefly, regions of interest (ROIs) for echo intensity measurements were drawn within the muscle following the contours of the muscle just below the fascia (figure E2). The lateral borders of the ROI were removed to exclude artifacts on the border of each image. The average echo intensity of the three images was used for analyses. The standard deviation (SD) of the average gray scale levels, as a measure of homogeneity, was analyzed as described before.[16] A more homogenous muscle may be caused by loss of muscle architecture due to muscle breakdown, inflammation or fluid retention.[16]

Muscle thickness and echo intensity can be age, gender, dominance, length and weight dependent.[25] Therefore, raw ultrasound values were converted to z-scores using normal values which were acquired from published healthy populations[25,26] and a new healthy population (see table E2 for the regression model formulas and table E3 for the characteristics of the new healthy control population). The z-scores represent the distance between the raw ultrasound value and the healthy population mean in units of the standard deviation. A z-score is negative when the raw score is below the population mean and positive when above.

For nerve assessment, a 6-18 MHz linear array transducer was used with constant image acquisition settings. Focus was adjusted according to nerve depth. Intraneural vascularization was investigated with power Doppler (low pulse repetition frequency 500 Hz, frequency 11.1 MHz, Doppler gain adjusted until random noise was encountered and then lowered until the noise disappeared, low persistence[27]). The median nerve was assessed at the wrist and 7 cm proximally and the peroneal nerve at the fibular head and at the popliteal fold, in a transversal and longitudinal plane. On transverse images, the cross-sectional area was measured within the hyperechogenic rim. Nerve diameter thickness was assessed on longitudinal images.

Clinical data collection

We collected the following clinical characteristics: age, sex, body weight and length at ICU admission, hand dominance, admission type, admission diagnosis, Acute Physiology and Chronic Health Evaluation IV (APACHE IV) score, maximal total Sequential Organ Failure Assessment (SOFA) score and presence of sepsis (according to the Bone criteria[28]) before inclusion. In addition, we collected data on pre-existing polyneuropathy or myopathy, risk factors for polyneuropathy before ICU admission (diabetes mellitus, alcohol abuse, chemotherapy, kidney failure), days with mechanical ventilation, ICU length of stay and ICU mortality.

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Sample size estimation

Sample size calculation was based on results of one study, in which echo intensity of the TA muscle increased by a factor 1.2 between 0 and 14 days after ICU-admission.[16] We used the standardized echo intensity of the TA for males (33.9 SD 9.3)[25] and multiplied this value by factor 1.2, giving 40.8, again assuming a SD of 9.3. Thirty patients per group were required to detect a difference of 6.9 (40.8-33.9) with 80% power and a two-sided alpha level of 0.05. Incidence of ICU-AW in patients mechanically ventilated for >48 hours in our institution is around 50%.[29,30] To account for technically imperfect data, we aimed to include at least 70 patients.

Statistical analysis

Normally distributed values are presented as mean with SD, non-normally distributed values as median with interquartile range (IQR), and proportions with percentages and total numbers. Differences between normally distributed continuous variables were assessed using Welch's t test, and between non-normally distributed continuous variables using Wilcoxon rank-sum test. Pearson's chi-square or Fisher exact test was used to assess differences between proportions.

As a measure of variability, the median coefficient of variation (CV) of three analyses of echo intensity per muscle was assessed.

Discriminative power of NMUS was assessed using receiver operating characteristic (ROC) curves with calculated area under the curve (AUC) with 95% confidence interval (CI). Discriminative power of AUC values between 90 and 100% was defined as excellent, between 80 and 90% as good, between 70 and 80% as fair, between 60 and 70% as poor, and <60% as failed.

Secondly, sensitivity and specificity and positive and negative predictive values (PPV, NPV) for muscle thickness and echo intensity were calculated based on a z-score cut-off of -2 for thickness parameters and +2 for echo intensity. We chose this cut-off point since 95% of the z-score values of healthy people lie between -2 and +2. Since weakness in ICU-AW is diffuse, we also investigated a composite outcome of amount of muscles with a thickness z-score of -2, or echo intensity of +2.

Analyses were done using R version 3.1.2 and R studio with the following packages: plyr, pROC, caret, tableOne, ggplot2.

RESULTS

From 1 September 2013 to 1 June 2015, a total of 76 patients gave informed consent and 71 were available for analysis, of whom 41 had ICU-AW. See figure 1 for the flowchart of screening and inclusion. Patient characteristics are presented in table 1.

```
┌─────────────────────────────┐
│ 3237 Patients admitted to the │
│            ICU              │
└─────────────────────────────┘
              │
┌─────────────────────────────┐
│ 541 Newly admitted patients and │
│   > 48 hours on MV screened    │
└─────────────────────────────┘
              │        ┌──────────────────────────────────────────┐
              │        │ 294 Patients excluded:                    │
              │        │    Stroke: 110                            │
              │        │    Cardiac arrest: 82                     │
              │        │    Traumatic brain injury: 47             │
              │        │    Neuromuscular disorder: 11             │
              │        │    Modified Rankin score >3: 11           │
              │────────│    Spinal injury: 9                       │
              │        │    Cognitive impairment: 6                │
              │        │    Intracerebral infection: 6             │
              │        │    < 1 arm and 1 leg available for NMUS or │
              │        │    muscle strength testing: 5             │
              │        │    Intracerebral space-occupying lesion: 4 │
              │        │    Moribund: 3                            │
              │        └──────────────────────────────────────────┘
┌─────────────────────────────┐
│ 247 Patients eligible for inclusion │
└─────────────────────────────┘
              │        ┌──────────────────────────────────────────┐
              │        │ 171 Patients no informed consent:          │
              │        │    Died: 56                               │
              │────────│    Transferred before awake and attentive: 43 │
              │        │    No consent: 33                         │
              │        │    Ongoing delirium: 20                   │
              │        │    No Dutch: 14                           │
              │        │    Missed: 5                              │
              │        └──────────────────────────────────────────┘
┌─────────────────────────────┐
│ 76 Patients informed consent │
└─────────────────────────────┘
              │        ┌──────────────────────────────────────────┐
              │        │ 5 Patients excluded from analysis          │
              │        │    Whitdrawal of informed consent: 1       │
              │────────│    Transferred before NMUS was done:1      │
              │        │    Violation of in-and exlusion criteria: 1 │
              │        │    Loss of NMUS data due to technical error: 2 │
              │        └──────────────────────────────────────────┘
┌─────────────────────────────┐
│ 71 Index and reference test   │
│         available            │
│                              │
│ 71 Muscles available          │
│    Biceps Brachii: 71         │
│    Flexor carpi radialis: 67  │
│    Rectus Femoris: 69         │
│    Tibialis Anterior: 70      │
│                              │
│ 70 Nerves available           │
│    Median nerve at carpal     │
│    tunnel: 68                 │
│    Median nerve proximal: 70  │
│    Peroneal nerve at fibular  │
│    head: 44                   │
│    Peroneal nerve at knee     │
│    fold: 57                   │
└─────────────────────────────┘
```
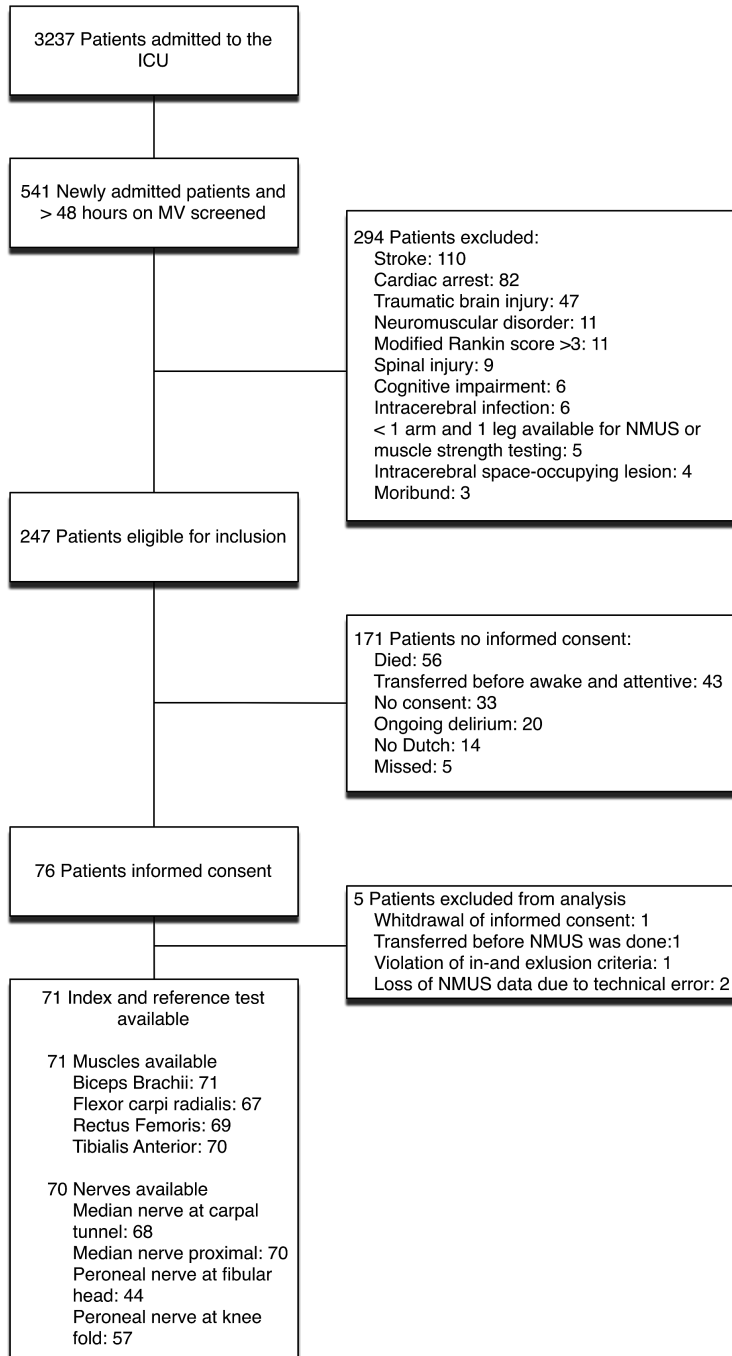
8

Figure 1. Flowchart of patient screening and inclusion
ICU=intensive care unit; MV=invasive mechanical ventilation; NMUS=neuromuscular ultrasound.

Table 1. Patient characteristics

	No ICU-AW N=30	ICU-AW N=41	P-value
Male (%)	22 (73)	25 (61)	0.41
Age (median years [IQR])	62 [49-69]	60 [51-70]	0.80
Body length (median cm [IQR])	175 [170-180]	172 [168-183]	0.46
Body weight (median kg [IQR])	75 [70-81]	75 [65-90]	0.66
History of myopathy (%)	0 (0)	1 (2)	1.00
History of polyneuropathy (%)	0 (0)	0 (0)	1.00
Any polyneuropathy risk factor in history (%)	14 (47)	22 (54)	0.73
Diabetes mellitus (%)	2 (7)	5 (12)	0.69
Alcohol abuse (%)	5 (17)	10 (24)	0.56
Kidney failure(%)	2 (7)	1 (2)	0.57
Chemotherapy (%)	7 (23)	9 (22)	1.00
Admission reason (%)			0.87
Medical (%)	12 (40)	18 (44)	
Emergency surgical (%)	9 (30)	10 (24)	
Elective surgical (%)	9 (30)	13 (32)	
Dominant hand right (%)	26 (90)	34 (92)	1.00
APACHE IV score (median [IQR])	58 [50-77]	71 [61-85]	<0.01
Maximum total SOFA score before ultrasound (median [IQR])	8 [8-12]	12 [10-14]	<0.01
Sepsis before ultrasound (%)	18 (60)	32 (78)	0.17
Mean MRC (median [IQR])	4.4 [4.0-4.7]	3.5 [2.8-3.7]	NA
Day of MRC (median [IQR])	7 [4-11]	9 [6-14]	0.02
Day of NMUS (median [IQR])	7 [5-10]	9 [6-14]	0.02
Length of MV (median days [IQR])	6 [3-8]	8 [5-17]	0.01
Length of stay on ICU (median days [IQR])	9 [6-13]	15 [9-23]	<0.01
Death in ICU (%)	0 (0)	2 (5)	0.51

IQR=interquartile range; APACHE=acute physiology and chronic health evaluation; SOFA=sequential organ failure assessment; MRC=medical research council; NMUS=neuromuscular ultrasound; MV=mechanical ventilation; ICU=intensive care unit.

Table 2. Univariate analysis of muscle thickness, echo intensity and echo intensity standard deviation (SD) and z-scores, and area under the receiver operating characteristic curve (ROC-AUC).

	No ICU-AW	ICU-AW	P-value	ROC-AUC (95% CI)
Thickness				
BB thickness (mean cm (SD))	2.6 (0.6)	2.2 (0.5)	0.002*	68.0 (55.4-80.7)
FCR thickness (mean cm (SD))	1.1 (0.2)	1.0 (0.2)	0.035*	64.5 (50.4-78.7)
RF thickness (mean cm (SD))	3.1 (0.9)	2.8 (0.8)	0.258	55.6 (41.8-69.4)
TA thickness (mean cm (SD))	2.2 (0.4)	2.1 (0.4)	0.558	54.3 (40.3-68.3)
Z-score thickness				
BB z-score thickness (mean (SD))	0.1 (0.6)	-0.3 (0.5)	0.011*	66.7 (53.7-79.6)
FCR z-score thickness (mean (SD))	-0.7 (1.2)	-1.3 (0.9)	0.033*	65.5 (51.3-79.7)
RF z-score thickness (mean (SD))	-0.5 (0.7)	-0.6 (0.8)	0.656	53.2 (39.4-67.1)
TA z-score thickness (mean (SD))	-0.4 (0.7)	-0.4 (1.0)	0.818	51.3 (37.5-65.1)
Echo intensity				
BB absolute echo intensity (mean gray scale level (SD))	76.2 (14.1)	79.1 (16.4)	0.434	57.6 (44.0-71.3)
FCR absolute echo intensity (mean gray scale level (SD))	62.3 (12.6)	67.7 (13.6)	0.095	63.2 (49.6-76.9)
RF absolute echo intensity (mean gray scale level (SD))	77.2 (17.0)	83.9 (14.8)	0.094	60.2 (46.4-74.0)
TA absolute echo intensity (mean gray scale level (SD))	88.6 (12.4)	94.6 (12.1)	0.046*	60.8 (47.4-74.1)
Z-score echo intensity				
BB z-score echo intensity (mean (SD))	0.5 (1.4)	0.7 (1.9)	0.519	56.3 (42.8-69.9)
FCR z-score echo intensity (mean (SD))	1.4 (1.6)	1.9 (2.0)	0.207	59.2 (45.2-73.2)
RF z-score echo intensity (mean (SD))	1.0 (1.6)	1.6 (1.8)	0.137	58.7 (45.0-72.4)
TA z-score echo intensity (mean (SD))	1.0 (1.2)	1.5 (1.4)	0.118	59.4 (45.9-72.9)
Echo intensity SD				
BB echo intensity SD (mean gray scale level(SD))	29.0 (4.0)	28.5 (4.4)	0.585	53.3 (39.6-67.0)
FCR echo intensity SD (mean gray scale level(SD))	21.3 (3.1)	20.8 (3.9)	0.573	56.2 (42.2-70.2)
RF echo intensity SD (mean gray scale level(SD))	24.2 (3.3)	23.5 (3.5)	0.398	55.9 (42.0-69.8)
TA echo intensity SD (mean gray scale level(SD))	25.8 (3.6)	26.2 (3.9)	0.698	54.4 (40.5-68.3)
Z-score echo intensity SD				
BB z-score echo intensity SD (mean (SD))	0.3 (1.5)	0.1 (1.5)	0.746	53.3 (39.6-67.0)
FCR z-score echo intensity SD (mean (SD))	0.8 (1.4)	0.6 (1.7)	0.716	55.0 (41.0-69.1)
RF z-score echo intensity SD (mean (SD))	0.1 (1.6)	0.04 (1.4)	0.890	54.2 (36.9-65.5)
TA z-score echo intensity SD (mean (SD))	-0.3 (1.1)	-0.2 (1.4)	0.777	55.0 (41.1-68.9)

BB=biceps brachii; FCR=flexor carpi radialis; RF=rectus femoris; TA=tibialis anterior.

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Muscle ultrasound

Thickness and thickness z-scores were lower in BB and FCR muscles in ICU-AW patients compared to patients without ICU-AW (Table 2 and Figure E3). However, discrimination between patients with ICU-AW and patients without ICUAW was poor for BB and FCR and failed for RF and TA thickness and all z-scores of thickness (Table 2).

For echo intensity, median CV of three analyses per muscle was 1.9-5.1%. Echo intensity was higher in TA muscle in ICU-AW patients, but z-scores of echo intensity were not different (Table 2). Discrimination between patients with ICU-AW and patients without ICU-AW based on echo intensity was poor and failed on z-scores of echo intensity.

Sensitivity, specificity, PPV and NPV based on z-score cut-off values are presented in Table 3. Specificity was high, but sensitivity, PPV and NPV were all low.

Echo intensity SD and z-scores did not differ between patients with and without ICU-AW and discrimination failed.

A composite outcome of amount of muscles with a thickness z-score of <-2 did not correctly classify patients (ROC-AUC 54.6% (95% CI 43.7-65.5%)), nor did the amount of muscles with an echo intensity z-score >2 (ROC-AUC 56.8% (95%CI 43.4-70.2%)).

Table 3. Diagnostic test evaluation for muscle z-score cut-offs
Sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) for z-score cut-offs of -2 for muscle thickness and +2 for echo intensity.

	Cut-off	Sensitivity	Specificity	PPV	NPV
BB thickness	Z-score <-2	3%	97%	50%	43%
FCR thickness	Z-score <-2	27%	83%	67%	48%
RF thickness	Z-score <-2	3%	97%	50%	43%
TA thickness	Z-score <-2	8%	93%	60%	43%
BB echo intensity	Z-score >2	36%	67%	58%	44%
FCR echo intensity	Z-score >2	46%	73%	68%	52%
RF echo intensity	Z-score >2	36%	67%	58%	44%
TA echo intensity	Z-score >2	38%	80%	71%	49%

BB=biceps brachii; FCR=flexor carpi radialis; RF=rectus femoris; TA=tibialis anterior.

Nerve ultrasound

CSA of the median nerve at the wrist was lower in ICU-AW patients, but showed poor discrimination (table 4). The other nerve CSA and thickness measures were not different and discrimination failed. Nerve vascularization did not differ between patients with and without ICU-AW.

Table 4. Univariate analysis and area under the receiver operating characteristic curve (ROC-AUC) of nerve parameters

	No ICU-AW	ICU-AW	P-value	ROC-AUC (95% CI)
Median nerve				
CSA wrist (mean mm^2 (SD))	10.7	8.9 (2.1)	0.020*	66.7 (53.6-79.9)
CSA proximal (mean mm^2 (SD))	7.3	7.6 (1.7)	0.445	52.5 (38.6-66.4)
Thickness proximal (mean mm (SD))	2.4	2.4 (0.4)	0.977	51.0 (37.2-65.2)
Intraneural vascularization proximal (%)[#]	6 (20.0)	10 (25.6)	0.793	
Peroneal nerve				
CSA fibular head (mean mm^2 (SD))	10.8	11.7 (5.0)	0.504	53.1 (35.6-70.7)
CSA knee fold (mean mm^2(SD))	8.2	8.2 (3.0)	0.999	52.4 (36.9-67.9)
Thickness knee fold (mean mm (SD))	2.2	2.3 (0.4)	0.505	54.6 (38.9-70.2)
Intraneural vascularization proximal (%)[#]	3 (12.0)	4 (14.3)	1.000	

*$p<0.05$, [#] in transversal or longitudinal plane. CSA=cross-sectional area

DISCUSSION

This study showed that the diagnostic accuracy of quantitative NMUS for diagnosis of ICU-AW is poor when assessed at awakening (median 7-9 days after ICU admission). A single or composite NMUS measurement cannot distinguish between patients with or without ICU-AW. For a z-score cut-off of -2 for muscle thickness and +2 for muscle echo intensity (corresponding to values found in 2.3% of healthy individuals), specificity was high, but sensitivity and PPV and NPV were low.

Muscle thickness and echo intensity

We found that thickness and z-scores of thickness of BB and FCR muscles were significantly lower in ICU-AW patients compared to patients without ICU-AW and echo intensity of TA was higher. However, there is a huge overlap of NMUS values of the two groups, causing low diagnostic accuracy. We also found that most z-scores were between -2 and +2, which is usually considered the normal range (corresponding to values found in 95% of healthy people).

More time may be needed for muscle thickness to decrease and for echo intensity to increase. Long-term studies show that increased time in the ICU is associated with a substantial reduction in muscle thickness (up to 17.7-30.4% at day 10 after ICU admission[17,21] and 38.9% after 4 weeks[19]). However, muscle atrophy is also seen in healthy volunteers after bed rest and might not discriminate between patients with and without ICU-AW.[31] Moreover, echo intensity may increase more slowly. The process of recovery of injured muscle tissue, giving an increase in fibrous and/or fat tissue in muscle, may not be detectable in the first weeks after initial muscle injury.[32] However, inflammation may be detectable earlier. In patients

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with severe sepsis, semiquantitatively graded echo intensity was already significantly higher at day 4 after admission compared to controls.[22]

To determine diagnostic accuracy we chose the moment of awakening, because it allowed a cross-sectional design with direct comparison to strength measurements. However, a diagnosis of ICU-AW before awakening (before muscle strength measurements are possible) is more desirable, because an early diagnosis is a prerequisite for any future preventive measure or treatment to be implemented. Since we found that diagnostic accuracy of NMUS at awakening was poor, it is less likely that differences in thickness and echo intensity will be more noticeable before this time point. Besides, muscle thickness and echo intensity can be influenced by confounding factors, like excessive fluid administration, often present in the first days after ICU admission, impairing the use of NMUS for early diagnosis of ICU-AW.

Alternatively, it might be that not the values at one time point, but the rate at which muscle size decreases or echo intensity increases can discriminate between ICU-AW and no ICU-AW. This would require multiple assessments with ultrasound in the first days after ICU admission to acquire an early diagnosis. Whether the changes in muscle thickness or echo intensity in the first days after admission would be evident enough to discriminate between ICU-AW and no ICU-AW is unknown. Studies with serial NMUS measurements within the first week after admission do not show uniform results: some studies showed a decrease in muscle mass at day 7 after ICU-admission, varying from 12.1%[15] to 6.0-24.9%[17], while others did not find changes from baseline in the first week after ICU admission[16,33]. Hence, the decrease in muscle thickness might be limited in the first week and might become more apparent thereafter. Increase in echo intensity is also more obvious after 7 days on the ICU.[16,17] Differences between patients with and without ICU-AW were not assessed in these studies.

Nerve ultrasound in the ICU

To our best knowledge, nerve ultrasound has never been investigated before in ICU patients. Diagnostic accuracy of nerve thickness or CSA for diagnosing ICU-AW in our study was poor. The CSA of the median nerve measured at the carpal tunnel at the wrist was smaller in ICU-AW patients, other nerve CSA or thickness parameters were not different. This at least indicates that nerve thickness is not increased in ICU-AW when compared to patients without ICU-AW, which is in line with an axonal neuropathy, since nerve thickness is often increased in demyelinating polyneuropathies.[8]

It is hypothesized that nerve damage in ICU-AW may be caused by increased vascular permeability causing endoneurial edema and subsequent hypoxia.[34] To compensate, perineural veins may dilate and cause hyperemia and hypervascularization, which could be detected by NMUS.[35] We found

hypervascularization in the median nerve in 16 patients and in the peroneal nerve in 7 patients. However, there were no differences between patients with and without ICU-AW.

Strengths and weaknesses

This is the first study investigating the diagnostic accuracy of quantitative NMUS for diagnosing ICU-AW, and also the first study ever to investigate quantitative nerve ultrasound in ICU patients. We used muscle strength testing as the reference standard, which is a clinically relevant reference standard and recommended method to diagnose ICU-AW.[4] A large number of patients was included and muscles and nerves were investigated with ultrasound in a systematic and reproducible way. The use of z-scores made the comparisons of measurements more reliable because age, gender and muscle side can influence muscle thickness and echo intensity.

As a first step to determine diagnostic accuracy we investigated NMUS parameters only at the first time point on which the reference standard (MRC score) was available. A limitation is that we did not perform NMUS measurements before or after that time point. We can therefore not rule out that a change in thickness or echo intensity over time may have a good diagnostic accuracy. Additionally, we did not investigate muscle CSA as a measure of muscle mass in our study. Furthermore, NMUS and strength assessment was performed approximately two days later in ICU-AW patients compared to patients without ICU-AW. As there was more time in the ICU-AW group for muscle thickness to decrease and echo intensity to increase, this may have potentially overrated the difference with the group without ICU-AW. Given the already low diagnostic accuracy found, this only strengthens our conclusions. Additionally, it might be that muscles that were not assessed in our study may be more sensitive to changes in thickness or echo intensity. Moreover, muscle biopsy or electrophysiological recordings were not performed in our study, because these are not routinely performed in clinical practice to diagnose ICU-AW in our ICU. Therefore, we cannot discriminate between CIP, CIM and CINM in this study.

Although the investigators performing the NMUS were blinded for the exact MRC scores, total blinding to muscle strength is not possible, since the presence or absence of spontaneous movements of the patient already gives an impression of the muscle strength. Additionally, except for intra-rater CV of echo intensity measurements, we did not assess inter- and intra-rater variability.

A limitation of quantitative NMUS in general is the fact that it is complicated to directly compare NMUS results between studies, because of differences in image acquisition settings, probe position, ultrasound machines etc. Gray scale levels are specific for the ultrasound machine used and cannot be compared to data obtained

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by other ultrasound machines unless calibrated.[8] Calibration can be done with a universal phantom.

CONCLUSION

A single neuromuscular ultrasound at the moment a patient awakens does not discriminate between patients with and without ICU-AW.

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COMPETING INTERESTS

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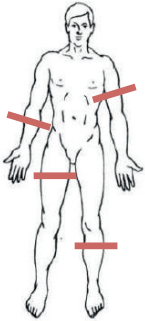
DATA SUPPLEMENT

Figure E1. Muscle measurements predefined measurement sites
If ultrasound was not possible on the preferred side, for example due to arterial lines or dressings, the opposite side was studied.

Table E1. Muscle measurements predefined measurement sites

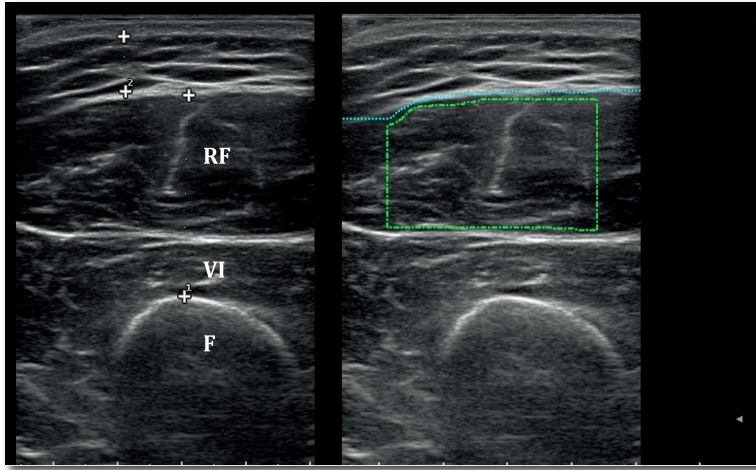|	Biceps Brachii (BB)	Flexor carpi radialis (FCR)	Rectus femoris (RF)	Tibialis anterior (TA)
Preferred side Measurement site	Left 2/3 distance from acromion to antecubital crease	Right 1/3 distance from antecubital fold to distal radius (muscle has triangular shape)	Right Halfway from anterior superior iliac spine and superior border of patella	Left 1/3 distance from inferior border of patella to the lateral malleolus
Correct transducer position	Humerus located in the middle and neurovascular bundle on the edge of the picture	Horizontal and flat position of radius and ulna. 15˚ angle between radius and ulna	Femur located in the middle with vastus intermedius and rectus femoris visible above the femur	Tibial cortex at about 45˚ angle. Central fascia is evident
Muscle thickness measurement	From humerus to upper border of BB (including brachialis muscle)	From deepest point of FCR to upper border of FCR	From femoral bone to upper border of RF (including vastus intermedius muscle)	From lower border of TA to upper border
Caliper positions for thickness and subcutaneous tissue				
Region of interest; lateral borders removed automatically				

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Figure E2. Ultrasound image of the rectus femoris
Ultrasound image of the rectus femoris (RF) muscle with calipers placed for muscle thickness (including the vastus intermedius muscle (VI)) and subcutaneous tissue (left picture), and region of interest (ROI) drawn (right picture). F=femur.

Table E2. Regression model formulas for normal values of muscle thickness and muscle echo intensity

Muscle	Sex	Age	Side	Regression model thickness	Regression model echo intensity
BB	M	18-99	D	$0.415+0.047*A-8.07*10^{-4}*A^2+3.79*10^{-6}*A^3-0.071*S$	$64.337+3.617*10^{-5}*A^3$
			Non-D	$0.415+0.047*A-8.07*10^{-4}*A^2+3.79*10^{-6}*A^3-0.071*S$	$48.311+0.4*A$
	F	18-99	D	$0.754+1.68*10^{-4}*A^2-1.68*10^{-4}*A^3-0.081*S$	$61.432+3.391*10^{-3}*A^2$
			Non-D	$0.754+1.68*10^{-4}*A^2-1.68*10^{-4}*A^3-0.081*S$	$62.594+3.853*10^{-5}*A^3$
FCR	M	18-99	D	$0.762+0.007*W$	$46.951+2.192*10^{-5}*A^3$
			Non-D	$0.825+0.007*W$	$40.965+0.179*A$
	F	18-81	D	1.11	57.197
		81-99	D	1.11	$46.951+2.129*10^{-5}*A^3$
	F	18-81	Non-D	$0.388+0.03*BMI$	56.727
		81-99	Non-D	$0.388+0.03*BMI$	$10.965+0.179*A$
RF	M	18-25	Both	$1.518-8.31*10^{-7}*A^3$	55.827
		25-99	Both	$1.518-8.31*10^{-7}*A^3$	$47.917+0.332*A$
	F	18-99	Both	$3.985-0.005*A-0.015*L$	$81.507+0.283*A-0.357*W$
TA	M	18-99	Both	$0.959-1.9*10^{-7}*A^3$	$59.65+0.301*A$
	F	18-99	Both	2.16	$67.17+0.29*A$

BB=biceps brachii; FCR=flexor carpi radialis. RF=rectus femoris; TA=tibialis anterior; M=male; F=female; D=dominant; Non-D=non-dominant; A=age in years; S=side (right=1; left=2); W=weight in kg; BMI=body mass index; L=length in cm.
Thickness regression models for BB and TA muscles were acquired from Arts et al. (Muscle and Nerve 2010) and the thickness regression models of RF muscles from Nijboer et al. (Muscle and Nerve 2011).
Thickness regression models of FCR and all echo intensity regression models were acquired from a new healthy control cohort from the Radboud University Medical Center Nijmegen (see table E3).

Table E3 Characteristics new healthy control cohort
Characteristics of new healthy control cohort of Radboud University Medical Center Nijmegen

| Age group	Number		Height (cm)		Weight (kg)		Body Mass index (BMI)	
	M	F	M	F	M	F	M	F
18-30	6	7	184.0 (182.3-187.3)	167.0 (166.0-169.0)	74.0 (72.3-78.8)	60.0 (54.5-62.0)	21.9 (21.0-23.1)	22.0 (19.6-22.7)
30-40	4	5	186.5 (183.3-187.8)	173.0 (168.0-176.0)	75.0 (72.0-82.8)	70.0 (60.0-72.0)	22.6 (21.4-24.6)	22.9 (21.6-24.1)
40-50	6	3	180.0 (180.0-183.8)	174.0 (170.5-182.0)	77.0 (74.8-77.0)	69.0 (67.0-93.5)	23.8 (23.1-24.3)	24.7 (23.1-28.7)
50-60	6	7	176.5 (172.8-178.8)	171.0 (165.5-172.0)	69.5 (68.3-71.5)	66.0 (61.0-68.0)	23.0 (21.9-24.0)	22.5 (22.0- 23.2)
60-70	4	7	179.0 (174.5-182.3)	171.0 (160.0-172.8)	95.5 (84.0-99.8)	76.0 (69.5-82.0)	29.2 (26.1-30.9)	25.1 (24.6-28.3)
70-80	4	5	183.0 (175.3-184.5)	170.0 (168.0-172.0)	83.5 (76.0-85.8)	71.0 (69.0-74.0)	24.4 (24.2-25.1)	24.4 (23.7-25.6)
>80	2	2	166.5 (166.3-166.8)	176.0 (174.5-177.5)	70.5 (68.7-72.3)	77.5 (76.3-78.8)	25.4 (24.9-26.0)	25.1 (24.2-25.9)

M=male; F=Female.

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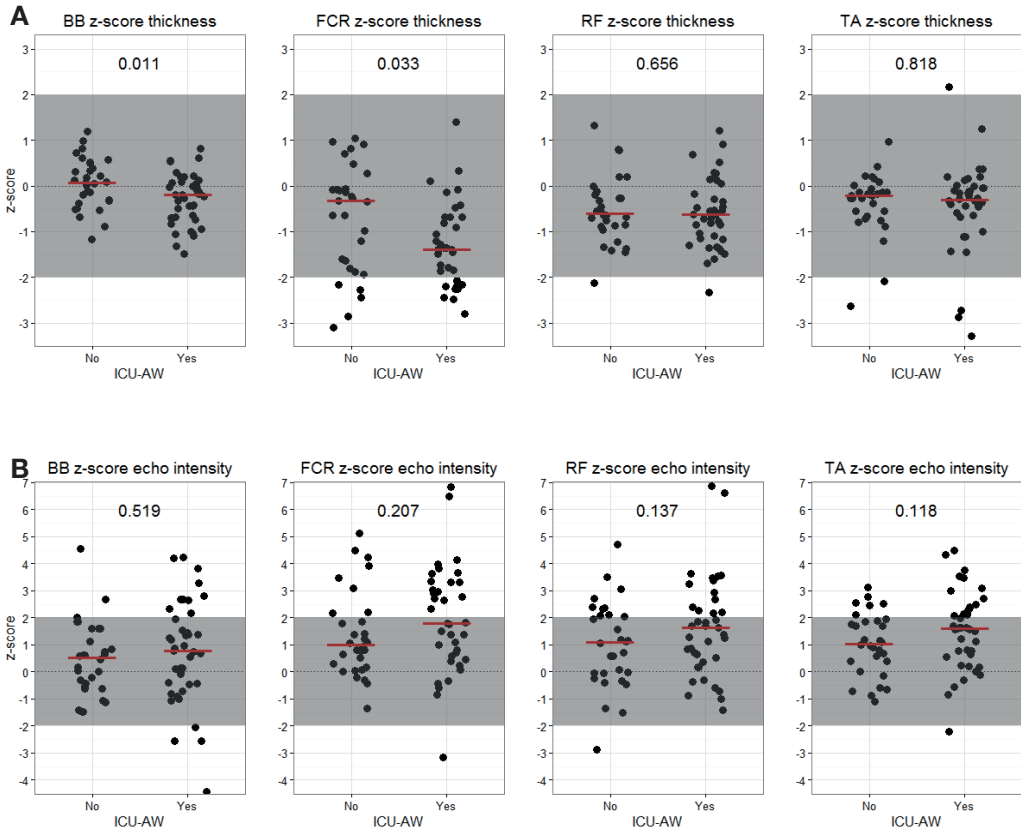
Figure E3. Muscle thickness and echo intensity
Z-scores of muscle thickness (A) and echo intensity (B) for four muscles. Crossbars represent mean values. The shaded area is the area from z-score -2 to +2, the area in which 95% of the values of healthy people are. Differences are analyzed using Welch's t test and p-values are expressed.
BB=biceps brachii; FCR=flexor carpi radialis; RF=rectus femoris; TA=tibialis anterior; ICU-AW=ICU-acquired weakness.

CHAPTER 9

**Early prediction of intensive care unit-acquired weakness:
a multicenter external validation study**

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ABSTRACT

Introduction

An early diagnosis of intensive care unit-acquired weakness (ICU-AW) is often not possible due to impaired consciousness. To avoid a diagnostic delay we previously developed a prediction model, based on data derived from one ICU, to predict ICU-AW at two days after ICU admission. The objective of this study was to investigate the external validity of the original prediction model and, if necessary, to update the model.

Methods

We performed a multi-center prospective observational cohort study at ICUs of five Dutch hospitals. Newly admitted ICU patients who were mechanically ventilated at 48 hours after ICU admission were included. Predictors were prospectively recorded and the outcome ICU-AW was defined by an average medical research council (MRC) score <4. In this validation cohort we analyzed performance of the original prediction model by assessment of calibration and discrimination. Additionally, we updated the model and evaluated a new prediction model based on combined data of the development and validation cohort.

Results

One hundred and ninety out of 349 analyzed patients developed ICU-AW. Both model calibration and discrimination of the original model were poor in the validation cohort. The area under the receiver operating characteristics curve (AUC-ROC) was 0.60 (95% confidence interval (CI) 0.54-0.66). Model updating methods improved calibration but not discrimination. The new model, developed using the combined data from the development and validation cohort had a fair discrimination, AUC-ROC 0.70 (95% CI 0.66 - 0.75), which was comparable to that of the original model in the development study.

Conclusions

The previously developed prediction model for ICU-AW showed poor performance in a new independent validation cohort. Model updating methods improved calibration but not discrimination. A newly derived prediction model showed fair discrimination.

This indicates that early prediction of ICU-AW is still challenging and needs further attention.

INTRODUCTION

Intensive care unit acquired weakness (ICU-AW) is a frequent complication of critical illness and is associated with prolonged stay in the ICU and increased short- and long term morbidity and mortality.[1-3] Before structural muscle and nerve damage is detectable, muscle and nerve dysfunction occurs, which may be fully reversible.[4,5] Therefore, future treatments may be most beneficial early in the disease. Furthermore, early detection of ICU-AW is relevant because it provides accurate prognostic information and enables timely initiation of supportive interventions, like tracheostomy and intensive physiotherapy.[6,7]

ICU-AW is currently diagnosed by assessment of manual muscle strength.[8] This is often not possible in the first couple of days after ICU admission because of impaired consciousness or attentiveness. To avoid this diagnostic delay we previously developed a prediction model for ICU-AW, including three early available predictors obtained two days after ICU admission.[9] The model showed fair discriminative performance after internal validation, but was built on data collected in only one hospital.

Before prediction models can be applied in practice, the external validity should be studied in a new independent population.[10] The aim of this study was to externally validate and, if necessary, update the previously developed prediction model for ICU-AW. External validation included both temporal (patients from a later time period) and geographical (patients from other institutions) validation to assess generalizability of the model.

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METHODS

Design and ethical approval

We performed a multicenter prospective observational cohort validation study. This study was reported according to the recently published TRIPOD (transparent reporting of a multivariable prediction model for individual prognosis or diagnosis) guidelines.[11,12]

The institutional review board of the Academic Medical Center, Amsterdam, the Netherlands, decided that the Medical Research Involving Human Subjects Act does not apply to this study (decision notice W13_193#13.17.0239) and therefore written informed consent was not needed. Verbal consent to use patient data was obtained from all included patients. The study was registered in the Netherlands Trial Register (#NTR4331).

Study setting

The study was conducted in medical-surgical ICUs of five hospitals in the Netherlands: two university hospitals, two university affiliated teaching hospitals and one regional hospital.

In- and exclusion criteria

Consecutive, newly admitted ICU patients, mechanically ventilated at 48 hours after ICU admission were included (irrespective of the duration of mechanical ventilation). This was different from the development study where patients who were mechanically ventilated for >2 days after admission were included. As in the development study, we excluded patients with an admission diagnosis of cardiac arrest, central nervous system (CNS) disease (stroke, traumatic brain injury, CNS infection or CNS tumor) or neuromuscular disease as well as patients with pre-existing spinal injury, poor pre-ICU functional status (modified Rankin scale ≥4)[13] and poor prognosis (expected to die within 48 hours).

Predictor assessment

All 20 candidate predictors of the development study[9] were assessed. These predictors were defined, collected and interpreted as in the model development study, except for lowest P/F ratio which was defined by the lowest PaO2 in the first 48 hours divided by the FiO2 on the concurrent time point (instead of the lowest of all P/F ratios in the development cohort).

Additionally, three new candidate predictors, based on newly described risk factors were collected: erythrocyte transfusion[14], hypercalcemia[15] and hypophosphatemia (own data, not published). These were defined as: any erythrocyte transfusion within 24 hours before ICU admission or in the first 48 hours after ICU admission, highest ionized calcium (mmol/L) and lowest phosphate (mmol/L) in the first 48 hours after ICU admission, respectively.

All predictors were prospectively assessed and recorded in an online case report form by local investigators, blinded for the strength assessment results.

Strength assessment (reference standard)

As in the development study, trained physiotherapists assessed muscle strength as soon as patients were alert (Richmond Agitation and Sedation Scale between -1 and 1) and attentive (able to follow verbal commands using facial expressions).[16-18] Muscle strength was assessed using the Medical Research Council (MRC) score in 6 pre-specified muscle groups, as in the development study.[9,19] The average MRC score was used for the analysis (values were not imputed when a muscle group could not be assessed). ICU-AW was defined by an average MRC score <4, in

accordance with international consensus statements.[1,8] Physiotherapists were blinded for the predictors (except age, gender, admission reason).

Additional data collected

We additionally collected the following clinical characteristics: the Acute Physiology and Chronic Health Evaluation IV (APACHE IV) score, the maximal Sequential Organ Failure Assessment (SOFA) score of the first two days after ICU admission, day of MRC assessment, number of days on mechanical ventilation, length of stay in the ICU and ICU mortality.

Data analysis

We applied the original model, with its predictors and assigned weights as estimated in the development study[9], to our new data. The original model was:

$$P_{ICUAW} = \frac{e^{-2.7763+0.0212*Age+0.7324*Highest\ Lactate+0.9506*Treatment\ any\ aminoglycoside\ (=yes)}}{1+e^{-2.7763+0.0212*Age+0.7324*Highest\ Lactate+0.9506*Treatment\ any\ aminoglycoside\ (=yes)}}$$

We assessed the performance by calibration and discrimination. Calibration reflects the agreement between the predicted ICU-AW risk by the model and the observed ICU-AW frequency in the validation cohort. This was assessed for each decile of predicted risk, ensuring 10 equally sized groups, by calculating the ratio of predicted ICUAW risk to observed ICUAW frequency. Calibration was analyzed graphically and using goodness of fit (Hosmer-Lemeshow test). Discrimination, the ability of the test to correctly classify those with and without the disease, was assessed by the area under the receiver operating characteristic curve (AUC-ROC). We defined AUC-ROC values between 0.90-1 as excellent, 0.80-0.90 as good, 0.70-0.80 as fair, 0.60-0.70 as poor and <0.60 as failed.

Next, to improve the performance of the original model, we used updating methods, which combine the information that is captured in the original model with the information of the new patients, instead of making a whole new prediction model. These previously described updating methods[20], vary from simple re-calibration (re-estimation of the intercept or slope of the linear predictor) to more extensive revisions, like re-estimation of some or all regression coefficients and model extension with new predictors. Before step-wise addition to the model, distributions of the three new candidate predictors were checked for normality. AUC-ROCs of the updated models were calculated. The change in Akaike Information Criterion (AIC) between the updated models and the recalibrated model were compared.[21] A model in which the AIC was at least 2 points lower than the AIC of the recalibrated model, was considered an improved model. In this improved model the re-estimated predictors were shrunken towards the re-calibrated model,

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any new predictors were shrinked toward zero and the intercept was again determined.

To further assess improved discrimination we evaluated the degree of correct reclassification using the continuous net reclassification improvement (cNRI), which is more sensitive to change than the AUC-ROC.[22] The cNRI of the updated model was compared to the recalibrated model. We also assessed the cNRI of the APACHE IV score and maximal SOFA score in the first two ICU days.

As a sensitivity analysis to assess the influence of missing data, we examined calibration and discrimination in data sets in which missing data was imputed, using multivariate imputation by chained equations (10 iterations of 10 imputations).[23] All predictors and the outcome (ICU-AW) were used for the imputation model. We checked validity of imputed data. AUC-ROC and corresponding confidence intervals of the 10 imputed data sets were averaged using Rubin's rules, a method to take into account variation within and between multiple imputation data sets.[24]

A second sensitivity analysis assessed the influence of the difference in inclusion criteria between the developments study and the validation study. We repeated the analysis for patients who were two days mechanically ventilated at time of inclusion. As an additional sensitivity analysis we assessed the performance of the model in only the patients of the hospital in which the model was developed.

Furthermore, we used the combined data from the development and validation cohort to make a new prediction model. Predictor selection was done as comprehensively described in the development study.[9] In short, we used bootstrapped backward selection and selected those predictors that were selected in >50% of the bootstrap samples (n=1000, p<0.05). Calibration and discrimination were assessed.

Proportions are presented with percentages and total numbers, mean values with standard deviation (SD) and median values with interquartile range (IQR). Differences between proportions were assessed using chi-square test, differences between normally distributed variables using Welch's t-test and differences between non-normally distributed continuous variables using Wilcoxon rank-sum test. Test results are presented with corresponding 95% confidence intervals (CI). Analyses were done using R (version: 3.3.1).

Power calculation

Empirical evidence suggests a minimum of 100 events and 100 non-events for external validation studies.[25] With an incidence of ICU-AW of about 50%, at least 200 patients were needed for validation (and updating). To further validate an updated model, 200 additional patients would be needed. We aimed to include at least 500 patients to account for people in whom MRC measurements could not be

performed (i.e. because they died before it could be measured, had an ongoing delirium etc.)

RESULTS

Screened and included patients

Figure 1 displays the flow chart. Consecutive ICU patients were screened for inclusion from February 2014 to December 2015. A total of 538 patients fulfilled the inclusion criteria and did not have any exclusion criteria. In 349 patients muscle strength could be assessed, of whom 190 patients had ICU-AW and 159 did not have ICU-AW. Because the loss of patients was larger than accounted for, we decided to use all available patients for the external validation of the original model and updating.
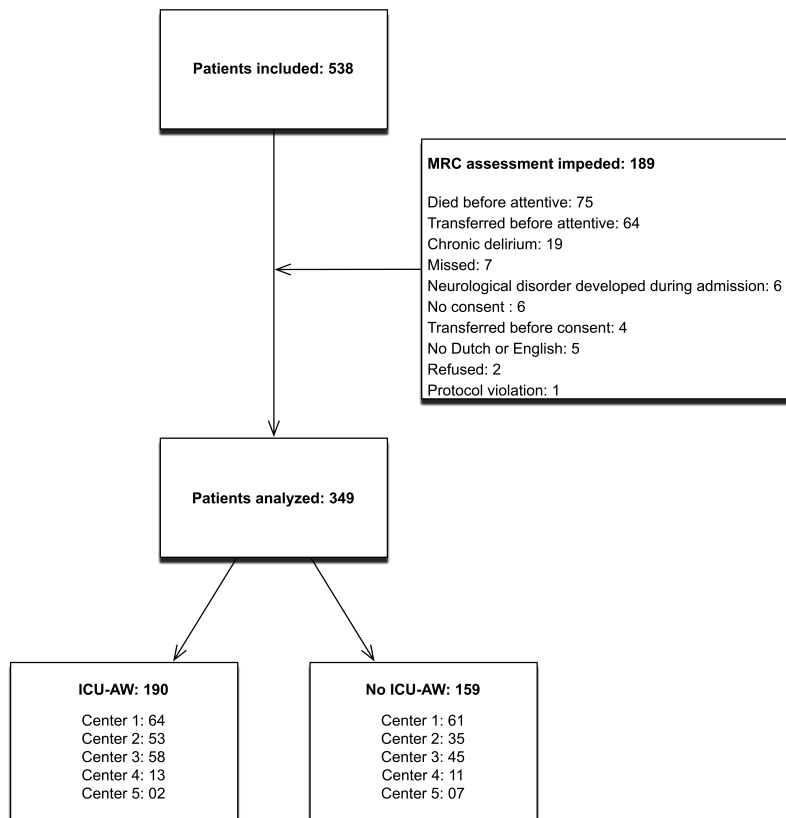

Fig 1. Flowchart of screened and included patients.
Center 1 is the center in which the original model was developed.
ICU-AW=intensive care unit-acquired weakness; MRC=medical research council.

Relatedness between the development and external validation cohorts

Table 1 shows the study and patient characteristics of the development and validation study. In the validation study, incidence of ICU-AW was slightly higher than in the development study (55% vs 49%), although the difference was not statistically significant. There were significant differences between the validation cohort and the development calibration cohort regarding maximal SOFA score in the first two days, day of MRC assessment and number of days with mechanical ventilation.

Table 2 shows the distribution of the assessed predictors of the development and external validation cohort. Patients from the validation cohort had significantly less often sepsis, less urine production, lower P/F ratios, higher platelet counts, higher ionized Ca^{2+}, less repeated treatment with any neuromuscular blocker and less treatment with any aminoglycoside. Also, highest glucose levels were lower and lowest glucose levels were higher.

Performance of the original model in the validation cohort

The original model was applied to our validation cohort. Calibration was poor with evidence for lack of fit (figure 2). The predictions were too extreme: for low predicted probabilities by the model the true fraction with ICU-AW was higher and for high predicted probabilities the true fraction was lower. The AUC-ROC was 0.60 (95% CI 0.54-0.66), which is interpreted as poor discrimination.
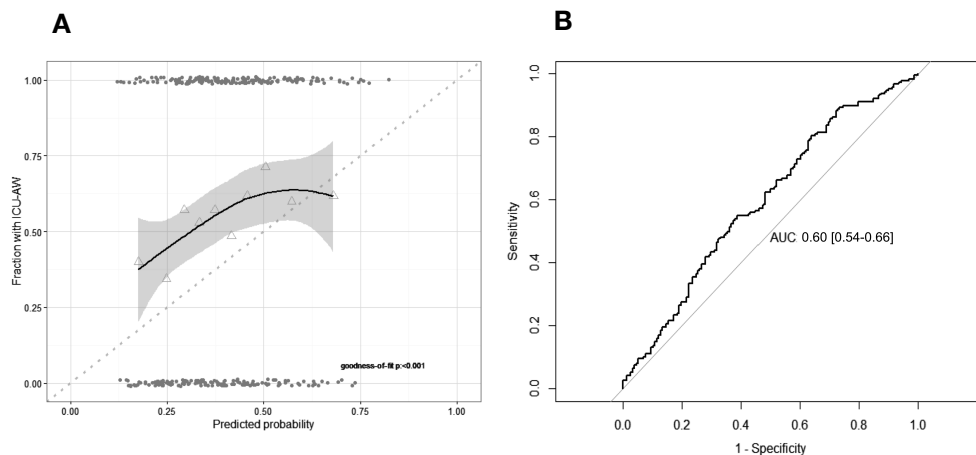

Fig 2. Model performance: calibration and discrimination of original model
Panel A shows the model calibration assessed with a fitted curve based on loess regression with 95% confidence interval. Perfect calibration is illustrated by the dotted line. Triangles represent deciles of predicted probability, grey points represent predicted probabilities of individual patients. Goodness of fit was assessed with the Hosmer-Lemeshow test. Panel B shows model discrimination assessed with the receiver operating characteristic curve.
ICU-AW=Intensive care unit -acquired weakness; AUC=area under the curve.

Table 1. Study and patient characteristics

	Development cohort N=212	External validation cohort N=349	p-value
Data collection period	January 2011 to December 2012	February 2014 to December 2015	
Study design	Prospective observational cohort	Prospective observational cohort	
Setting	Mixed medical-surgical ICU of one academic medical center in the Netherlands	Mixed medical-surgical ICU's of 5 hospitals in the Netherlands	
Inclusion criteria	Consecutive, newly admitted ICU patients mechanically ventilated for ≥2 days	Consecutive, newly admitted ICU patients mechanically ventilated at 48 hours after ICU admission	
Outcome	Presence of ICU-AW	Presence of ICU-AW	
Reference standard	Average MRC score<4	Average MRC score<4	
Incidence of ICU-AW, n (%)	103 (49)	190 (55)	0.208
Age, mean ±SD	61 (16)	63 (14)	0.050
Females, n (%)	92 (43)	136 (39)	0.347
Reason for admission			0.994
planned surgical, n (%)	44 (21)	72 (21)	
emergency surgical, n (%)	49 (23)	85 (24)	
medical, n (%)	119 (56)	192 (55)	
APACHE IV score, mean ±SD	81 (28) (3 missing)	79 (27) (16 missing)	0.272
Maximal SOFA score in first two days, mean ±SD	10 (3)	9 (3) (12 missing)	0.013
Average MRC score, median [IQR]	4.0 [2.6, 4.8]	3.8 [3.2, 4.5]	0.834
Day of MRC assessment after ICU admission, median [IQR]	8 [6, 12]	6 [4, 10]	<0.001
Days with MV, median days [IQR]	8 [4, 16]	6 [4, 11]	0.001
LOS ICU, median days [IQR]	10 [7, 18]	9 [6, 17]	0.107
ICU mortality, n (%)	21 (10)	25 (7)	0.269

ICU-AW=intensive care unit-acquired weakness; LOS ICU=length of stay in the intensive care unit; APACHE IV=Acute Physiology and Chronic Health Evaluation IV; SOFA=Sequential Organ Failure Assessment; MV=mechanical ventilation; MRC=Medical Research Council; SD=standard deviation; IQR=inter quartile range.

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Table 2. Distributions of candidate predictors

Predictors	Development cohort (n=212)	External validation cohort (n=349)	p-value
Patient characteristics			
Females, n (%)	92 (43)	136 (39)	0.344
Age, mean (± SD)	*61 (16)*	*63 (14)*	*0.053*
Risk factor for a polyneuropathy in medical history, n (%)	75 (35)	147 (43) (9 missing)	0.082
Pre-existing polyneuropathy prior to ICU admission, n (%)	4 (2)	11 (3) (18 missing)	0.467
Systemic corticosteroid use prior to ICU admission, n (%)	16 (8)	25 (7) (12 missing)	1.000
Clinical parameters			
Suspected sepsis, n (%)	148 (70)	199 (57)	0.003
Unplanned admission, n (%)	168 (79)	277 (79)	1.000
Presence of shock, n (%)	142 (67)	222 (64)	0.472
RASS score, median [IQR]	-3 [-4, 0]	-2 [-4, -1] (6 missing)	0.388
Laboratory parameters			
Average urine production, median ml/h [IQR]	87 [40, 128]	64 [41, 98]	0.002
Highest glucose, mean mg/dl (± SD)	231.8 (73.7)	219.3 (63.9)	0.034
Lowest glucose, mean mg/dl (± SD)	87.8 (24.2)	103.5 (24.4)	<0.001
Lowest pH, mean (± SD)	7.23 (0.10)	7.23 (0.11)	0.790
Lowest P/F ratio, median [IQR]	180 [129, 246]	144 [96, 200] (1 missing)	<0.001
Lowest platelet count, median x10^9/L [IQR]	118 [66, 173]	150 [83, 221] (5 missing)	<0.001
Highest lactate, median mmol/L [IQR]	*3.7 [2.2, 6.0] (17 missing)*	*3.3 [2.1, 5.2] (2 missing)*	*0.087*
Lowest ionized Ca^{2+}, mean mmol/L (± SD)	0.98 (0.12)	1.03 (0.14)	<0.001
Highest ionized Ca^{2+}, mean mmol/L (± SD)		1.22 (0.12)	
Highest phosphate, mean mmol/L (± SD)		0.89 (0.37) (8 missing)	
Treatment			
Treatment with any corticosteroid, n (%)	144 (68)	244 (69.9)	0.689
Repeated treatment with any neuromuscular blocker, n (%)	35 (17)	33 (9.5)	0.019
Treatment with any aminoglycoside, n (%)	*81 (38)*	*48 (13.8)*	*<0.001*
Transfusion of erythrocytes, n (%)		132 (37.8)	

The predictors in *italic* are the predictors included in the original prediction model.
SD=standard deviation; IQR=inter quartile range; RASS=Richmond Agitation and Sedation Scale; P/F=PaO2/FiO2; Ca=calcium.

Model updating

We tried several methods to update our model (table 3 and figure 3) using recalibration, re-estimation and extension with new candidate predictors. Model updating, using method 6 in which the new candidate predictors were added one-by-one to the recalibrated model (method 3), improved discrimination when lowest phosphate was added to the model. With all updating methods calibration improved, but the AUC-ROC remained 0.60 (95% CI 0.54-0.66). The cNRI of the updated model was as good as the cNRI of the recalibrated model.
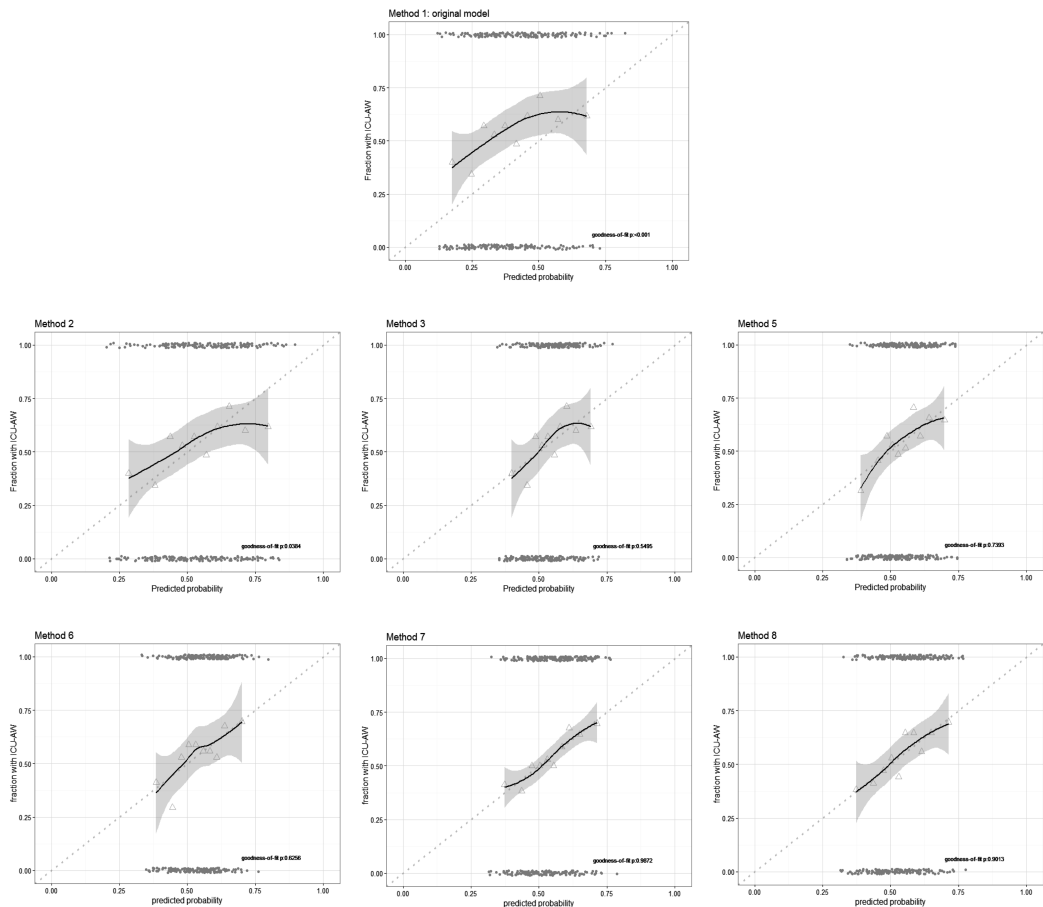

Fig 3. Calibration plots of updated models.
Model calibration of the updated models from table 3 were assessed with a fitted curve based on loess regression with 95% confidence interval. Perfect calibration is illustrated by the dotted line. Triangles represent deciles of predicted probability, grey points represent predicted probabilities of individual patients. Goodness of fit was assessed with the Hosmer-Lemeshow test.
ICU-AW=Intensive care unit acquired weakness.

Table 3. Model updating results
Method 1 is the original model. The model was recalibrated by adjusting only the intercept (method 2) or both the intercept and slope (method 3). With method 4 we investigated whether predictors were having a clearly different effect in the validation cohort, by selective re-estimation of one or more of the included predictors. None of the models with re-estimations improved the model, therefore no selective re-estimations were done. In method 5, the model was fitted in the validation data by re-estimation of the intercept and regression coefficients for all predictors. In method 6 the three new predictors were one-by-one added to the recalibrated model. Only adding lowest phosphate improved the model. In method 7, model 5 was extended with new predictors. In method 8, a model with all old and new predictors was assessed. Shrinkage was applied to the improved model (method 6) and the intercept was recalculated.

| | No updating | Re-calibration | | Model revision | | Model extension | | | Shrinkage |
	Method 1: original model	Method 2: update intercept	Method 3: re-calibration of intercept and slope	Method 4: re-calibration and selective re-estimation	Method 5: re-estimation	Method 6: re-calibration (and selective re-estimation) and selective re-extension	Method 7: re-estimation and selective extension	Method 8: re-estimation and extension	Model 6
Intercept	-2.776	-2.154	-1.049	-1.049	-0.849	-1.361	-1.119	-1.027	-1.115
Age	0.021*	0.021	0.011	0.011	0.006	0.011	0.004	0.004	0.011†
Highest lactate‡	0.732*	0.732	0.384	0.384	0.508	0.384	0.537	0.515	0.384†
Aminoglycoside	0.951*	0.951	0.498	0.498	0.275	0.498	0.172	0.175	0.498†
Lowest phosphate						0.347	0.403	0.384	0.070**
Erythrocyte transfusion								0.083	
Highest calcium								-0.06	
Hosmer-Lemeshow test	<0.001	0.038	0.550	0.550	0.739	0.265	0.837	0.549	0.208
AUC ROC	0.60	0.60	0.60	0.60	0.60	0.60	0.61	0.61	0.60
	[0.54-0.66]	[0.54-0.66]	[0.54-0.66]	[0.54-0.66]	[0.54-0.66]	[0.54-0.66]	[0.55-0.67]	[0.55-0.67]	[0.54-0.66]

‡transformed using the natural logarithm, *uniform shrinkage factor applied, †shrinkage towards re-calibrated values, **shrinkage towards zero.
AUC ROC=area under the receiver operating characteristic curve.

Comparison with SOFA and APACHE IV scores

The AUC-ROC of the maximal SOFA score in the first 2 days after admission for prediction of ICU-AW in the validation cohort was 0.63 (95% CI 0.58-0.69) and the AUC-ROC of the APACHE IV score was 0.63 (95% CI 0.57-0.69). Compared to using the SOFA score, the updated model reduced classification with 31% (cNRI)(95% CI 9-52), whereas it performed as good as the APACHE IV score (21% (95% CI -1-43)).

Sensitivity analysis

Of the predictors used in external validation and updating analyses, highest lactate levels were missing in 2 patients and lowest phosphate in 8 patients; the other predictors included in the original model did not have missing values. The combined AUC-ROC of the imputed datasets was 0.59 (95% CI 0.53-0.65).

When the original model was only applied to patients who were mechanically ventilated for two days at the time of inclusion (n=291) the AUC-ROC was 0.58 (95% CI 0.51-0.64) and when it was applied to the patients in the hospital of the development study (center 1, n=123) the AUC-ROC was 0.59 (95%CI 0.49-0.69).

New prediction model

The following predictors were included in >50% of the bootstrap samples: RASS score, gender, highest lactate, lowest P/F ratio, highest glucose and ICU treatment with corticosteroids. In the final model (based on 536 patients due to missing values of RASS score (n=6) and lactate (n=19)) RASS, gender, highest lactate and treatment with corticosteroids were included (selected by a drop in AIC >2). A universal shrinkage factor (0.94) was applied to adjust for overfitting. The new prediction model is described with the following formula:

$$P_{ICUAW} = \frac{e^{-1.5724-0.2233*RASS\ SCORE+0.5699*gender\ (female=1)+\ 0.5107*Highest\ Lactate+0.4631*Treatment}}{1+e^{-1.5724-0.2233*RASS\ SCORE+0.5699*gender\ (female=1)+\ 0.5107*Highest\ Lactate+0.4631*Treatment}}$$

Calibration was excellent (figure 4). The AUC-ROC after internal validation was 0.70 (95% CI 0.66 - 0.75).

Discrimination improved when using the new prediction model compared to the SOFA or APACHE IV score (cNRI 38% (95% CI 21-55) and 30% (95% CI 13-47) respectively).
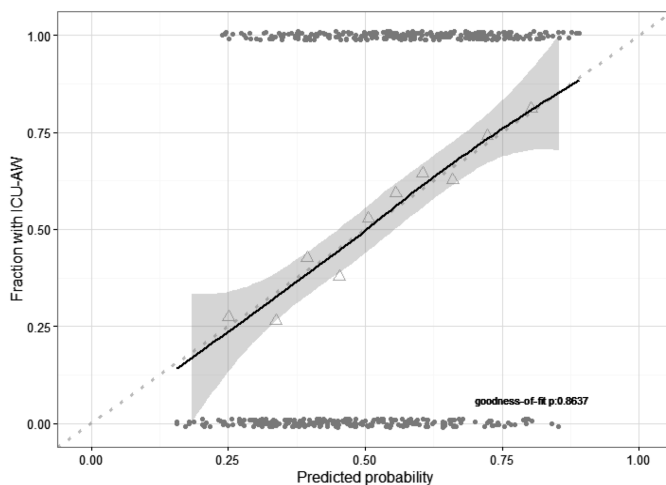
Fig 4. Calibration plot of new model
Calibration plot of new model based on combined data of the development and validation cohort. Model calibration was assessed with a fitted curve based on loess regression with 95% confidence interval. Perfect calibration is illustrated by the dotted line. Triangles represent deciles of predicted probability, grey points represent predicted probabilities of individual patients. Goodness of fit was assessed with the Hosmer-Lemeshow test.
ICU-AW=Intensive care unit acquired weakness.

DISCUSSION

In this study we assessed the performance of a previously developed prediction model for ICU-AW. The model showed both poor calibration and discrimination in our new patient cohort. Updating methods improved calibration, but not discrimination. A new prediction model based on combined data from the development and validation cohort had an excellent calibration. The AUC-ROC of this new model was 0.70 after internal validation. The new prediction model classified patients better than the SOFA and APACHE IV score.

Reasons for poor performance of the model

Poor performance in new datasets is often seen and can have several reasons.[12] First of all, this can be caused by differences in case-mix. The distribution of baseline characteristics and predictors showed differences between the development and the validation cohort. Patients in the validation cohort seemed to be less severely ill indicated by lower SOFA scores, less often sepsis, less days of mechanical ventilation, less repeated administration of neuromuscular blockers, and earlier MRC assessment whereas on the other hand these patients had less urine production and lower P/F ratio's. A major difference in use of aminoglycosides was seen, because it was regularly used in the center in which the model was

developed, but less frequent in the other centers. The differences in case mix cannot solely be explained by the multicenter design, since these differences were also seen when the development cohort was compared with validation cohort patients only from the hospital in which the model was developed. Although no major changes in standard of care were noted, unrecognized changes in care over time may cause differences in case mix, which could be a reason for failed temporal validation.

Besides differences in subject-level characteristics as described above, differences in study-level characteristics (such as inclusion criteria) can also lead to a worse performance. Our inclusion criteria in the validation cohort differed slightly from the development cohort, possibly selecting less severe patients because some patients had a lower duration of mechanical ventilation in the first 48 hours after admission. We chose for this inclusion criterion to make inclusion more easy for the investigators and to increase the amount of eligible patients. We assumed that this population would be comparable to the population in the development study. Actually, sensitivity analyses showed that when the original model was applied to only those patients who were mechanically ventilated for a duration of two days at inclusion (83% of the patients in the validation cohort), as in the development cohort, calibration and discrimination remained poor. Thus, differences in inclusion criteria do not explain the poor performance.

At last, the fact that the performance of the original model could not be reproduced in the validation cohort may be attributable to the small sample size of the development study causing unstable predictions and possibly incorrect predictor selection. In fact, in the newly developed model, including the cohorts of the development and validation cohort, other predictors (except for lactate) were selected.

Importance of external validation

The performance of any prediction model tends to be lower than expected when it is applied to new patients.[26] Therefore, every developed prediction model should be validated in new individuals before the model is applied in practice or implemented in guidelines. This step is, erroneously, often skipped.[12]

This study underlines the importance of external validation. It showed that generalizability and transportability of the previously developed model was poor and that the original model could thus not be used in clinical practice, also after extensive updating. Even the maximal SOFA score in the first 2 days after admission could predict ICU-AW better than the updated models.

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Limitations of the study

This study has some limitations not previously declared. Of all patients in which strength could not be measured (n=189), 64 patients were transferred before they were attentive. As the clinical condition of these patients allowed a transfer from the ICU to the ward, this group may be less severely ill and may contain less patients with ICU-AW, masking the true incidence of ICU-AW in the validation cohort.

Furthermore, because strength measurements were available in less patients than beforehand accounted for, we did not have enough data to validate the model, update the model and again externally validate an updated or new model. Therefore we used all available data to validate and update the model. Future studies should account for more loss of patients (due to dead, transfer, delirium etc.)

Development of a new prediction model

Model updating did not result in a useful model with sufficient discrimination and therefore a new model was developed using the development and validation cohort together. This new model included RASS score, gender, highest lactate and treatment with corticosteroids as predictors. The new model was based on a much larger cohort than the original development cohort, resulting in more stable estimates. The AUC-ROC was fair (0.70 (95% CI 0.66 - 0.75)) and comparable with that of the original model. External validation is needed to prove performance and clinical usefulness in a new validation cohort.

Recently another prediction model for ICU-AW was proposed[27], including the following predictors: steroid therapy, intensive insulin therapy, number of days on mechanical ventilation, sepsis, renal failure and hematologic failure. This model, which was based on data of 4157 patients, at least 12 hours mechanically ventilated, in whom only 3% had ICU-AW, showed good discrimination (AUC-ROC 0.81 (95% CI 0.78-0.84). Calibration was, however, not reported and external validation was not performed. In this study, the incidence of ICU-AW was lower than expected, probably caused by the definition of ICU-AW used. These differences make comparison of the study results difficult.

CONCLUSIONS

External validation of a previously developed prediction model for ICU-AW showed poor calibration and discrimination. Updating methods improved calibration but not discrimination. A new prediction model using data from the development and validation cohort showed fair discrimination and classified patients better than the APACHE IV and the SOFA score. However, early prediction of ICU-AW, using clinical parameters, with good discrimination seems to be challenging.

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CHAPTER 10

Summary and general discussion

The pathophysiology of intensive care unit-acquired weakness (ICU-AW) is complex and probably multifactorial. Much remains to be understood regarding the pathophysiological processes leading to muscle weakness. Inflammation may play an essential role. In **part I** of this thesis, we explored local and systemic inflammation in ICU-AW. We summarized current knowledge on local and systemic inflammation in ICU-AW **(chapter 2)** and we systematically reviewed the literature on local muscle and nerve inflammation in ICU-AW, both in animal models for and patients with ICU-AW **(chapter 3)**. We hypothesized systemic hyperinflammation to play a role in ICU-AW. In a prospective observational cohort study, we compared patterns of inflammatory markers in the first four days after ICU admission in patients with and without ICU-AW (**chapter 4**). Furthermore, we investigated differences in systemic complement activation in the first week after ICU admission (**chapter 5**).

A considerable part of the currently available pathophysiological explanations are derived from animal models. These animal models have several limitations. A translatable and easily applicable ICU-AW animal model does not exist yet, but could be essential to further unravel the pathophysiology of ICU-AW and to test therapeutic interventions. In **part II,** we investigated if existing animal models of sepsis could serve as models of ICU-AW, using *in vivo* measured strength as our primary outcome. For this we used to existing murine models, an *E. coli* peritonitis model (**chapter 6**) and a *S. pneumoniae* pneumonia model (**chapter 7**).

ICU-AW is currently diagnosed by manual muscle strength testing.[1] Consequently, the diagnosis of ICU-AW is often delayed, because sedation or reduced attentiveness hamper muscle strength testing, especially in the first days after ICU admission.[2] An early diagnosis of ICU-AW, however, could lead to earlier initiation of supportive interventions, like intensive physiotherapy and tracheostomy, and early enrolling of patients in future clinical trials, potentially improving outcomes. In **part III**, new and innovative diagnostic methods for early diagnosing of ICU-AW were investigated. We examined the use of neuromuscular ultrasound, a non-invasive and harmless procedure, to diagnose ICU-AW **(chapter 8).** Furthermore, we investigated the external validity of our previously developed prediction model to predict ICU-AW at two days after ICU admission, in a multicenter prospective observational cohort study **(chapter 9)**.[3]

INFLAMMATION IN IN THE PATHOPHYSIOLOGY OF ICU-AW

Since the first descriptions of critically ill patients developing a polyneuropathy or myopathy in the 1970s, it was noticed that all these patients had severe sepsis and multiple organ failure.[4] In the following decennia, the relationship between ICU-AW and sepsis, systemic inflammatory response syndrome (SIRS) and multiple organ dysfunction syndrome (MODS), was further established. Several studies showed

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that these factors are the main risk factors for ICU-AW.[5] Sepsis is a multifaceted host response to an infection and involves early activation of both pro- and anti-inflammatory responses.[6] These responses become dysregulated, leading to life-threatening organ dysfunction.[6-8] Because of the strong association between MODS and ICU-AW, we may have to see muscles and nerves as just other failing organs in the spectrum of MODS. A common pathogenesis is thus plausible.

After reviewing the literature **(chapter 2 and 3),** we concluded that local and systemic inflammation in ICU-AW are not studied extensively. Cellular infiltrates have been found in muscle tissue in ICU-AW animal models, but in muscle and nerve biopsies of ICU-AW patients such infiltrates were often not found.[9-11] Thus, muscle and nerve damage may not be directly caused by local inflammatory cell infiltration. However, this damage may result from other components of the inflammatory response, such as inflammatory mediators. Several inflammatory mediators, including antigen presenting molecules, complement factors, cytokines and cell adhesion molecules, have indeed been found in plasma and muscle and nerve tissue of patients with ICU-AW.[11-14] Cytokines, complement membrane attack complex and antigen presenting molecules were also found on the vascular endothelium. This may cause endothelial cell activation, resulting in increased permeability of the vascular endothelium and capillary leakage.[11] Subsequently, this may give rise to edema and impaired tissue oxygenation, leading to tissue damage.[15,16] Endothelial cell activation also causes microvascular occlusions of neutrophils adhering to the capillary walls, which further impairs oxygen delivery to the tissue.[17] Organ damage in MODS is considered to be a consequence of this indirect damage, at least in part.[17]

Interestingly, some inflammatory mediators can be expressed by and also released from skeletal muscle; the so-called myokines.[18] It might be that, in critical illness, release of myokines, especially interleukin (IL)-6, may contribute to systemic inflammation, perpetuating and disturbing the systemic inflammatory response, possibly adding to organ failure and muscle and nerve damage.

The studies reviewed in **chapter 2 and 3** had major methodological limitations. Since most studies were case series or cohort studies without control ICU patients, the findings may not be specific for patients with ICU-AW and may apply to all ICU patients. To further investigate local inflammation in ICU-AW, muscle and nerve biopsies would be desired, but these are not easily obtainable on the ICU. Muscle and nerve biopsies are invasive procedures and are hampered by coagulopathy, which is often seen in critically ill patients. Furthermore, nerve biopsies are controversial because they can cause persistent sensory deficits. As a consequence, we looked further into systemic inflammation instead of local inflammation.

Inflammatory mediators in sepsis have multiple targets and cause effects through several parallel mechanisms and they regulate each other.[19] Therefore, when studying associations, advanced statistical procedures to take this complexity into account are needed, such as principal component analysis. In **chapter 4,** we found that systemic inflammation was increased in patients who develop ICU-AW compared to patients who do not develop ICU-AW. This finding was consistent when adjusted for confounders, like disease severity. IL-6, IL-8, IL-10 and fractalkine were identified to be the most important factors. The both pro- and anti-inflammatory acting cytokine IL-6, pro-inflammatory acting cytokine IL-8, and anti-inflammatory cytokine IL-10 also play an important role in the imbalanced inflammatory response in sepsis and MODS.[20] Fractalkine is a recently noticed inflammatory mediator, which can act both as adhesion molecule and as a soluble chemokine.[21] The exact way in which these mediators may contribute to muscle and nerve damage has to be elucidated in further research. This may also possibly open the way to ameliorate ICU-AW by blockage of specific systemic inflammatory pathways.

The complement system is another important part of the innate immune system. Since it is involved in the development of sepsis, SIRS and MODS, and in several acute inflammatory polyneuropathies and myopathies, it is an obvious suspect in the development of ICU-AW.[22,23] Depositions of the membrane attack complex (which result from the final pathway of complement activation) have been found in muscles of patients with ICU-AW.[11] In the pilot study in **chapter 5**, systemic complement levels were elevated in all ICU patients in the first seven days after ICU admission, but were not different between patients with or without ICU-AW. This does not per definition rule out that complement plays a role in the pathophysiology of ICU-AW. Expression of membrane complement regulatory proteins (which determine the sensitivity to complement-mediated injury) may be different in patients who develop ICU-AW, but this has to be investigated.[24] Besides, the measured systemic levels of complement may not reflect local activation.

ANIMAL MODELS FOR ICU-AW

Several animal models have been used to study muscle dysfunction in ICU-AW, but all these models have limitations. First of all, models are often of short duration and represent acute sepsis, instead of the more prolonged sepsis as seen in ICU patients.[25,26] Some models are even more distinct from critical illness in humans, for example models in which nerves are crushed.[27,28] Others have low consistency and reproducibility, e.g. the cecal ligation and puncture model.[25,26,29] Some models mimic ICU interventions, but are very laborious because they need continuous monitoring for numerous days, for instance porcine or rat models with several days of mechanical ventilation.[27,30-32] Surprisingly, *in vivo* strength measurements have been

scarcely performed, although weakness is the clinical hallmark of ICU-AW.[1] *In vivo* strength measurements, by way of grip strength and the rotated screen test were both used once to detect *in vivo* strength in an animal ICU-AW model.[33,34] Considering all these limitations, we started to search for a new animal model for ICU-AW.

We investigated whether established sepsis models were suitable as ICU-AW models using grip strength as the main outcome. First, we investigated an *E. coli* septic peritonitis model in young and old mice (**chapter 6**). Old mice may be more susceptible to a decline in grip strength, since age is a risk factor for ICU-AW.[35] We found a decline in grip strength in all groups, but no differences in grip strength between the infected and the control groups, both in young and old mice. Next, we did experiments with a *S. pneumoniae* sepsis mouse model (**chapter 7**). Even though the mice showed severe illness, clinical weakness of limb muscles could not be detected by grip strength, and electrophysiological measurements did not show signs of ICU-AW. We had to conclude that our models could not serve as ICU-AW models. In both experiments we saw a drop in grip strength in all groups, suggesting a learning effect. Therefore, grip strength testing may not be the best *in vivo* method to detect acute muscle weakness in awake mice.

Nevertheless, we did find diaphragmatic weakness, assessed by *ex vivo* contractility measurements of the diaphragm. We did not perform contractility measurements of limb muscle. Previous animal studies have shown that inflammation impaired diaphragmatic contractility but not limb contractility, suggesting that the diaphragm is more susceptible to systemic inflammation than limb muscle.[36,37] Furthermore, diaphragmatic dysfunction may be twice as common as limb weakness in ICU patients.[38]

Our animal experiments showed that, although mice develop severe illness, it is difficult to induce ICU-AW in mice. Mice can become severely ill with a substantial and rapid weight loss, but they recover quickly or they die, and there seems to be nothing between those two extremes. This is not comparable to humans, where patients who survive their critical illness have long-term functional disability and often do not completely recover.[39] This may suggest that mice, or rodents in general, are not the most suitable animals to use in an ICU-AW model. Other animals, such as pigs, may be better species, but they need facilities that are not widely available and lead to high costs. To conclude, finding a good animal model for ICU-AW remains a challenge.

EARLY DIAGNOSIS AND INDIVIDUAL PREDICTION OF ICU-AW

The most common method to diagnose ICU-AW is manual muscle strength testing using the Medical Research Council (MRC) score.[1,40] Other methods include handgrip strength, nerve conduction studies, electromyography, direct muscle

stimulation and muscle and nerve biopsy.[1,41] Handgrip strength may be an alternative for the MRC score, but it is uncertain if it is representative of overall weakness in critically ill patients.[42,43] Like manual muscle strength testing, it needs an attentive and cooperative patient. Electrophysiological recordings are feasible in the ICU and can diagnose ICU-AW.[44,45] However, the diagnostic properties of electrophysiological screening vary over time.[44] Diagnostic accuracy of electrophysiological recordings to early diagnose ICU-AW, that is before patients are awake and manual muscle strength testing is possible, is low.[44,46] Besides, the importance of electrophysiological abnormalities in the absence of weakness is unclear.[41] Electrophysiological recordings, including nerve conduction studies, electromyography and direct muscle stimulation (DMS) have several disadvantages like discomfort, interference from electronic equipment and high costs.[41] Furthermore, they are not readily available in many ICU's and coagulation disorders hamper myography and DMS.[46] Muscle and nerve biopsies are invasive and as it takes time before structural changes to muscle and nerve develop, they are not suitable for an early diagnosis of ICU-AW. To diagnose ICU-AW early, new diagnostic methods are needed.

Neuromuscular ultrasound is a non-invasive and easy to use bedside test on the ICU and is thus at an advantage compared to other muscle imaging techniques like MRI or CT. A reduction in muscle thickness and increase in echo intensity over time has been previously described in ICU patients, but the relationship to ICU-AW has been unknown.[47,48] We found that a single neuromuscular ultrasound at the time a patient awakens did not discriminate between patients with and without ICU-AW **(chapter 8)**. It may take more time before muscles or nerves show structural changes and before these changes can differentiate between patients with and without ICU-AW. This will, however, not allow an early diagnosis.

Clinical prediction models may be used to estimate the probability that patients have ICU-AW and may aid in clinical decision-making. An advantage of clinical prediction models is that they can be updated when new predictors, such as biomarkers, become available, improving the performance of the model. We previously developed a prediction model, based on data derived from one ICU, to predict ICU-AW at two days after ICU admission.[3] This model, including three early available predictors, showed fair discriminative performance after internal validation. However, before a prediction model can be applied in practice, external validity should be studied in a new independent population. The previously developed prediction model showed poor performance in the new independent validation cohort and updating did not improve discrimination (**chapter 9**). This may have been caused by differences in case mix between the development cohort and the validation cohort or by the small sample size of the development study causing unstable predictions and possibly incorrect predictor selection. Early prediction of

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ICU-AW, using clinical parameters, with good discrimination seems to be challenging.

FUTURE PERSPECTIVES

Each improvement of healthcare starts with understanding of a disease and this understanding starts with awareness. Insights into ICU-AW already have changed remarkably over the past decades. Indeed, in the past ICU-AW was considered to be an 'insignificant' side effect of critical illness, and it was believed that all patients would fully recover. Nowadays, it is recognized that ICU-AW has a long-term impact on survival and physical functioning. Still, awareness of ICU-AW should be further improved. As ICU-AW may be seen in the spectrum of organ failure, it deserves the same attention as other failing organ systems in critically ill patients. Patients with ICU-AW now fall between two stools as neither the intensivist nor the neurologist consider themselves specialists for this problem. Physiotherapists play an important role in diagnosing ICU-AW, but intensivists should be trained to deal with the implications of this diagnosis and should consult a neurologist if other causes of weakness are suspected.

An essential step for a better understanding of ICU-AW is early awareness for and documentation of muscle strength. Muscle strength, and thereby ICU-AW, is currently not routinely documented on ICUs, and as such, exact incidence rates are unknown. Better documentation will also improve and facilitate research. The MRC sum score (MRC-SS) is widely used in manual muscle strength testing, but has some methodological shortcomings, partly due to the ordinal nature of the scale. Handgrip dynamometry and a newly introduced 4-point scale to document muscle weakness may be superior to the MRC-SS, but have to be further validated before they can be used in clinical practice on the ICU.[49,50] Besides, these methods still need an attentive and cooperative patient, which delays the diagnosis. Other diagnostic methods, such as biomarkers, can possibly circumvent this requirement.

More insight into the pathophysiology of ICU-AW is needed. The role of systemic inflammation should be further explored. Gene expression studies may give more insight into involved inflammatory pathways.[6] If we are able to elucidate the pathophysiology of ICU-AW, the way is open for the development of specific diagnostic and therapeutic interventions.

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CHAPTER 11

Nederlandse samenvatting

Ongeveer de helft van de patiënten die opgenomen zijn op de intensive care (IC) ontwikkelt spierzwakte.[2,3] Deze spierzwakte wordt intensive care-verworven zwakte, ook wel IC-zwakte, genoemd. Het ontstaat door dysfunctie of schade van spieren, zenuwen, of een combinatie van beide.[1,2] Door de verbeterende zorg op de IC overleven steeds meer mensen hun IC-opname, maar daarmee neemt het aantal complicaties, waaronder IC-zwakte, helaas wel toe.

In het verleden werd IC-zwakte beschouwd als een onbelangrijke bijkomstigheid van ernstige ziekte. De gedachte was dat alle patiënten geheel herstelden. Tegenwoordig wordt het steeds duidelijker dat IC-zwakte ernstige lange-termijn gevolgen kan hebben. Veel patiënten met IC-zwakte herstellen niet volledig en hebben nog jaren na ontslag van de IC last van lichamelijke beperkingen.[4,5] Daarnaast is IC-zwakte geassocieerd met een langere beademingsduur, langer verblijf op de IC en in het ziekenhuis, en verhoogde sterftekans.[3-5]

Er is geen gerichte behandeling voor IC-zwakte. Interventies, zoals fysiotherapie, zijn vooral ondersteunend.[6]

DE ROL VAN INFLAMMATIE BIJ HET ONTSTAAN VAN IC-ZWAKTE

Het is nog onduidelijk hoe IC-zwakte ontstaat, maar waarschijnlijk spelen meerdere factoren een rol.[2,7,8] Als micro-organismen het lichaam binnendringen, komt er als onderdeel van de afweer een ontstekingsreactie op gang. Als deze reactie ontspoort, kan er een buitensporige en onevenwichtige ontstekingsreactie in het gehele lichaam ontstaan, die weefsels en organen kan aantasten.[9] Dit wordt sepsis genoemd.[10] Sepsis, het systemisch inflammatoir respons syndroom (SIRS) en multi-orgaanfalen zijn de belangrijkste risicofactoren voor het ontstaan van IC-zwakte.[3] Een ontspoorde ontstekingsreactie zou dus ook een rol kunnen spelen bij het ontstaan van IC-zwakte. Vanwege de sterke associatie tussen multi-orgaanfalen en IC-zwakte moeten we spieren en zenuwen misschien ook wel zien als falende organen in het spectrum van multi-orgaanfalen.

In **deel I** van dit proefschrift hebben we de rol van lokale en systemische ontsteking (inflammatie) in IC-zwakte beschreven. De huidige kennis hierover is samengevat in **hoofdstuk 2.** In een systematisch literatuuronderzoek in **hoofdstuk 3** is nagegaan wat bekend is over lokale inflammatie in zenuw- en spierweefsel bij IC-zwakte, zowel in diermodellen als bij patiënten met IC-zwakte. Het bleek dat de rol van lokale en systemische inflammatie bij IC-zwakte niet uitgebreid onderzocht is. Clusters van inflammatoire cellen worden gezien in spierweefsel in diermodellen van IC-zwakte, maar deze zijn vaak afwezig bij patiënten met IC-zwakte.[11-13] Verschillende inflammatoire mediatoren, waaronder antigeen presenterende moleculen, complement factoren, cytokines en adhesiemoleculen, zijn wel gevonden in plasma en spier- en zenuwweefsel van patiënten met IC-zwakte.[13-16] Deze mediatoren kunnen op indirecte wijze schade geven aan het weefsel.

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De studies die worden samengevat in **hoofdstuk 2** en **3** hadden veel methodologische tekortkomingen. De meeste studies beschreven alleen enkele casussen van patiënten met IC-zwakte of het waren cohortstudies zonder controle IC-patiënten. Daarom zijn de bevindingen mogelijk niet specifiek voor patiënten met IC-zwakte en zouden ze gevonden kunnen worden bij alle IC-patiënten.

We hebben ons in dit deel van het proefschrift verder gefocust op systemische inflammatie. In **hoofdstuk 4** is onderzocht of patiënten met IC-zwakte een ander patroon van systemische inflammatie hebben dan patiënten zonder IC-zwakte. Systemische inflammatie bleek verhoogd in patiënten met IC-zwakte, ook als er gecorrigeerd werd voor verstorende invloeden, zoals ernst van ziekte. De belangrijkste inflammatoire mediatoren waren interleukine (IL)-6, IL-8, IL-10 en fractalkine. Verder onderzoek is nodig om te kijken hoe deze inflammatoire mediatoren bijdragen aan spier- en zenuwschade.

Het complementsysteem is een belangrijk onderdeel van het aangeboren immuunsysteem en activatie van dit systeem speelt een belangrijke rol in het ontstaan van sepsis, multi-orgaanfalen en verschillende spier- en zenuwziektes.[17,18] Complementdeposities zijn gevonden in spieren van patiënten met IC-zwakte.[13] **Hoofdstuk 5** beschrijft een studie, waarin is onderzocht of er verschillen zijn in complementactivatieproducten in plasma tussen patiënten met en zonder IC-zwakte. Spiegels van complementactivatieproducten waren verhoogd in alle IC-patiënten, maar er waren geen verschillen tussen patiënten met en zonder IC-zwakte.

DIERMODELLEN VOOR IC-ZWAKTE

Diermodellen kunnen essentieel zijn om het ontstaansmechanisme van IC-zwakte verder te ontrafelen en om toekomstige therapieën te testen. Huidige diermodellen voor IC-zwakte hebben echter veel tekortkomingen. Sommige modellen zijn erg kortdurend en daardoor niet vergelijkbaar met de aanhoudende sepsis zoals in IC-patiënten wordt gezien.[19,20] Daarnaast zijn modellen soms totaal niet vergelijkbaar met de situatie in mensen, bijvoorbeeld modellen waarin zenuwen kapot worden gedrukt.[21,22] Andere modellen zijn niet consistent of reproduceerbaar, zoals een model waarin gaten in de darm worden gemaakt.[19,20,23] In sommige diermodellen wordt de IC situatie wel goed nagebootst, maar deze modellen kosten veel tijd en geld, omdat ze continue bewaking nodig hebben. Rat- en bigmodellen met mechanische beademing gedurende meerdere dagen zijn hier een voorbeeld van.[21,24-26] Het is opvallend dat in al deze modellen bijna nooit *in vivo* kracht wordt gemeten, terwijl krachtsverlies het belangrijkste kenmerk van IC-zwakte is.

In **deel II** van dit proefschrift is onderzocht of bestaande muismodellen van sepsis gebruikt kunnen worden als model voor IC-zwakte. *In vivo* grijpkracht was onze belangrijkste uitkomstmaat. In **hoofdstuk 6** wordt een *E.coli* sepsis model met

jonge en oude muizen beschreven. Bij alle muizen werd een afname in kracht gevonden, maar geen verschillen tussen de geïnfecteerde muizen en de controlemuizen. In **hoofdstuk 7** onderzochten we een *S. pneumoniae* sepsis muismodel. Hoewel de muizen erg ziek werden, was er geen krachtsverlies. Electrofysiologische testen toonden ook geen afwijkingen aan zenuwen of spieren in de poten. We concludeerden daarom dat onze modellen niet als model voor IC-zwakte konden fungeren. Er werd overigens wel diafragmazwakte bij *ex vivo* contractiliteitsmetingen vastgesteld in het *S.pneumoniae* sepsismodel. *Ex vivo* contractiliteitsmetingen van andere spieren zijn niet gedaan.

Onze dierexperimenten tonen aan dat het, ondanks dat de muizen erg ziek werden, moeilijk is om IC-zwakte in muizen te induceren.

VROEGE DIAGNOSE EN INDIVIDUELE PREDICTIE VAN IC-ZWAKTE

De diagnose IC-zwakte wordt gesteld door het meten van de spierkracht van de ledematen.[1,27] Dit kan alleen als de patiënt wakker en alert genoeg is. IC-patiënten worden echter vaak gesedeerd, vooral in de eerste dagen van hun IC-opname en daardoor kan de kracht lange tijd niet gemeten worden.[28] Als een gevolg hiervan kan de diagnose IC-zwakte vaak pas laat gesteld worden. Een vroege diagnose is echter wel wenselijk, omdat dan eerder gestart kan worden met ondersteunende maatregelen, zoals intensieve fysiotherapie of het plaatsen van een tracheostoma zodat patiënten kunnen ontwennen van de beademing. Ook voor het includeren van patiënten in toekomstige studies is een vroege diagnose van belang.

In **deel III** van dit proefschrift hebben we nieuwe en innovatieve methoden onderzocht om IC-zwakte vroeg te kunnen diagnosticeren. In **hoofdstuk 8** wordt beschreven of neuromusculaire echografie IC-zwakte relatief vroeg kan diagnosticeren. Neuromusculaire echografie is een niet-invasieve en eenvoudige methode die aan het bed van de IC- patiënt kan worden toegepast. In IC-patiënten is een afname in spierdikte en toename in echo-intensiteit (grijswaarde) beschreven, maar de relatie met IC-zwakte was onbekend.[29,30] In onze studie bleek dat een eenmalige neuromusculaire echometing op het moment dat de patiënt ontwaakt niet kan discrimineren tussen patiënten met en patiënten zonder IC-zwakte. Neuromusculaire echografie kan daarom niet gebruikt worden voor een vroege diagnose van IC-zwakte.

In het verleden is met patiëntendata uit het AMC een model ontwikkeld om IC-zwakte te voorspellen na de eerste twee dagen IC-opname.[31] Dit model bestond uit drie voorspellende factoren en had een redelijke diagnostische nauwkeurigheid. Voordat een predictiemodel in de praktijk gebruikt kan worden moet het eerst aangetoond worden dat het model ook werkt in een nieuwe onafhankelijke populatie. In **hoofdstuk 9** hebben we de externe validiteit van ons predictiemodel onderzocht in een prospectieve cohortstudie met patiënten uit meerdere

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ziekenhuizen in Nederland. In deze nieuwe populatie had het predictiemodel een slechte diagnostische nauwkeurigheid. Verschillende methoden om het model te updaten verbeterden de discriminatie helaas niet. Het predictiemodel is dus niet goed genoeg om in de dagelijkse praktijk op de IC te gebruiken.

Vroege predictie van IC-zwakte met een goede diagnostische nauwkeurigheid lijkt een uitdaging te zijn.

TOEKOMSTPERSPECTIEVEN

Elke vooruitgang in de gezondheidszorg begint met kennis over een ziekte en om die kennis te vergroten is aandacht voor een ziekte noodzakelijk. Hoewel de lange-termijn gevolgen van IC-zwakte steeds beter bekend worden, schiet de aandacht voor IC-zwakte nog steeds te kort.

Een belangrijke stap om IC-zwakte beter te begrijpen is betere documentatie van spierkracht. Spierkracht, en daarmee IC-zwakte, wordt momenteel niet standaard gedocumenteerd op de IC en daarom is onbekend hoeveel patiënten IC-zwakte krijgen. Betere documentatie verbetert en vergemakkelijkt ook het wetenschappelijk onderzoek naar deze ziekte.

In de studies beschreven in dit proefschrift vonden we dat neuromusculaire echografie niet gebruikt kon worden om IC-zwakte vroeg te diagnosticeren en dat ons eerder ontwikkelde predictiemodel bij externe validatie niet goed bleek te zijn. Onderzoek naar nieuwe methoden om IC-zwakte vroeg te detecteren is daarom nog steeds onmisbaar. Hierbij zou bijvoorbeeld naar biomarkers gekeken kunnen worden.

Meer inzicht in de ontstaanswijze van IC-zwakte is noodzakelijk. We vonden dat systemische inflammatie verhoogd is in patiënten met IC-zwakte. De rol van systemische inflammatie moet verder uitgezocht worden. Gen expressiestudies kunnen mogelijk meer inzicht geven in de betrokken inflammatoire systemen. Diermodellen kunnen ook behulpzaam zijn bij studies naar de pathofysiologie van IC-zwakte. De onderzochte sepsis diermodellen in dit proefschrift bleken echter niet geschikt als model voor IC-zwakte.

Als we de ontstaanswijze van IC-zwakte kunnen ontrafelen, is de weg geopend voor de ontwikkeling van nieuwe diagnostische en therapeutische interventies.

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APPENDICES

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About the Author

Esther Witteveen was born on February 25[th] 1986 in De Meern, the Netherlands. In 2004 she graduated from high school at the Utrechts Stedelijk Gymnasium and started her medical training at the University Medical Center Utrecht (UMCU).

During her medical training she was already early interested in medical research and neurology. She participated in a research project on subarachnoid hemorrhages and cardiac arrhythmias and a project on ALS-associated genes. She also participated in multiple research projects of the UMCU as a research nurse. As elective internships she did rotations at the department of Rehabilitation and the department of Genetics at the UMCU and she did an internship in the Holy Family Hospital in Berekum, Ghana. After completion of her medical training in 2010, she started working as a resident in Neurology (not in training) at Tergooi Ziekenhuizen Blaricum.

In 2012, Esther started with her PhD project on the department of Intensive Care of the Academic Medical Center (AMC) in Amsterdam, witch lead to this thesis (PhD supervisors prof. dr I.N. van Schaik and prof. dr. M.J. Schultz, co-supervisors J. Horn and C. Verhamme). During her PhD project she also participated in the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) project, a large multicenter project. In March 2015 she started her residency in Neurology at the AMC (prof. dr. Y.B.W.E.M. Roos and prof. dr. I.N. van Schaik).

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List of publications

1. **Witteveen E**, Wieske L, van der Poll T, van der Schaaf M, van Schaik IN, Schultz MJ, Verhamme C, Horn J, Molecular Diagnosis and Risk Stratification of Sepsis (MARS) Consortium. Increased early systemic inflammation in ICU-acquired weakness; A prospective observational cohort study. Crit Care Med 2017:1.

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Witteveen E, Schultz MJ, Horn J. The role of local and systemic inflammation in the pathogenesis of intensive care unit-acquired weakness. Annual Update in Intensive Care and Emergency Medicine 2015;15:509-518. Springer International Publishing.

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PhD portfolio

Name PhD student: Esther Witteveen
PhD period: 01-06-2012 to 29-11-2017
Name PhD supervisor: Ivo N. van Schaik and Marcus J. Schultz
Name PhD co-supervisors: Janneke Horn and Camiel Verhamme

	Year	Workload (ECTS)
General and specific courses		
AMC World of Science	2012	0.7
Basic laboratory safety	2012	0.4
Laboratory animals	2012	3.9
Advanced immunology	2013	2.9
Good clinical practice (BROK)	2013	0.9
Practical biostatistics	2014	1.1
Clinical Epidemiology: Observational Epidemiology	2014	1.0
Informed consent training	2014	0.2
Seminars, workshops and master classes		
Master class complement	2012	0.2
Muscles2Meet symposium	2016	0.5
Presentations		
Inflammation in intensive care unit-acquired weakness: A systematic review. Poster at the Biennial Meeting of the Peripheral Nerve Society.	2013	0.5
Role of complement in ICU-acquired weakness. Oral presentation at master class complement.	2013	0.5
Neurofilaments as a plasma biomarker for ICU-acquired weakness: an observational pilot study. Oral presentation at MARS biennial Meeting.	2013	0.5
Intensive care unit-acquired weakness: grip strength does not decline in an E. coli peritonitis mouse model. Poster at the Annual congress of the European Society of Intensive Care Medicine.	2013	0.5
Early electrophysiological diagnosis of ICU-acquired weakness. Poster at 34th International Symposium on Intensive care and Emergency Medicine.	2014	0.5
Early complement activation in ICU-acquired weakness: a pilot study. Poster at Annual congress of the European Society of Intensive Care Medicine.	2014	0.5
Increased early systemic inflammation in patients with ICU-acquired weakness. Poster at 35th International Symposium on Intensive care and Emergency Medicine.	2015	0.5
Increased early systemic inflammation in patients with ICU-acquired weakness. Poster at Biennial Meeting of the Peripheral Nerve Society.	2015	0.5
Diagnostic accuracy of quantitative neuromuscular ultrasound for the diagnosis of intensive care unit-acquired weakness: a cross-sectional observational study. Poster at Muscles2Meet symposium Princess Beatrix Muscle fund.	2016	0.5

Diagnostic accuracy of quantitative neuromuscular ultrasound for the diagnosis of intensive care unit-acquired weakness: a cross-sectional observational study. Poster at meeting Dutch Society of Clinical neurophysiology.	2016	0.5
Diagnostische waarde van kwantitatieve neuromusculaire echografie voor de diagnose intensive care zwakte: een cross-sectionele observationele studie. Oral presentation at annual research meeting Dutch Society of Neurology.	2016	0.5
Diagnostic accuracy of quantitative neuromuscular ultrasound for the diagnosis of intensive care unit-acquired weakness. Oral presentation at the 3rd congress of the European Academy of Neurology.	2017	0.5

Conferences

Attendance biennial meeting of the Peripheral Nerve Society, St Malo, France	2013	1.0
Attendance annual congress of the European Society of Intensive Care Medicine, Paris, France	2013	0.75
Attendance annual International Symposium on Intensive Care and Emergency Medicine, Brussels, Belgium	2014	0.75
Attendance annual congress of the European Academy of Neurology, Amsterdam, the Netherlands	2017	1.0

Lecturing

ICU-acquired weakness. Training for intensivists.	2014	0.1

Other

Intensive care journal club (monthly)	2012-2015	2.5
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Bursary for participation congress of European Academy of Neurology, Amsterdam	2017	

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You can't play a symphony alone, it takes an orchestra to play it (Navjot Sigh Sidhu). En zo is het ook met promoveren. Je kunt het niet alleen en het is alleen maar mogelijk met de hulp van vele anderen. Daarom veel dank aan iedereen die op de een of andere manier heeft bijgedragen aan de totstandkoming van dit proefschrift. Ik wil een aantal mensen in het bijzonder bedanken.

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Esther