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Life after Intensive Care - Post-intensive care syndrome, inflammation, and HMGB1

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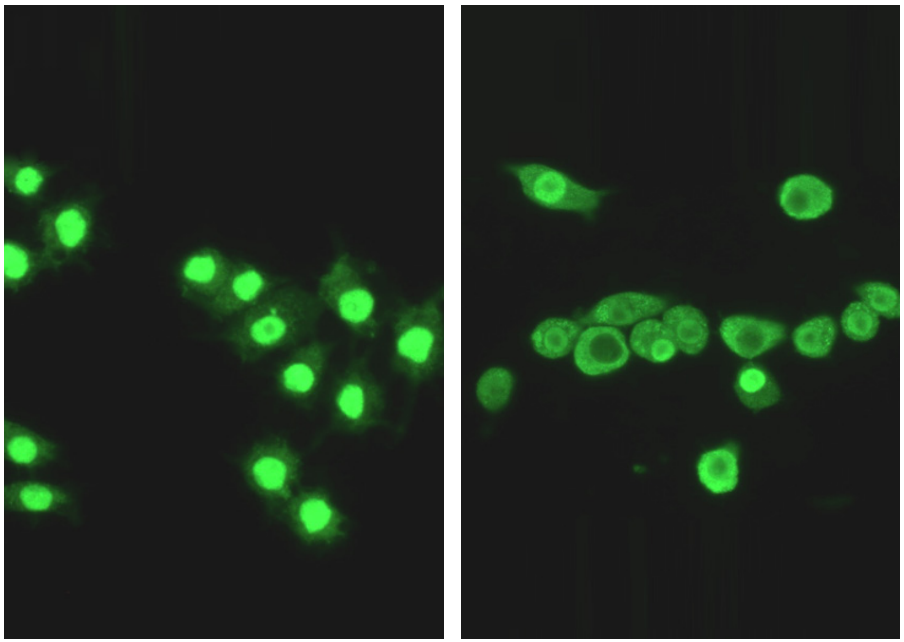

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LIFE AFTER INTENSIVE CARE - POST-INTENSIVE CARE SYNDROME, INFLAMMATION, AND HMGB1

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To my family, with endless love and gratitude

“It does not matter how slowly you go as long as you do not stop.” - *Confucius*

ABSTRACT

Survival rates of intensive care unit (ICU) patients are consistently increasing. This new patient population has recently gained increased recognition as they suffer from psychological, physical, and cognitive impairments in the years to come after ICU discharge. These symptoms are collectively referred to as the post-intensive care syndrome (PICS). Importantly, the underlying pathophysiology of PICS is unclear, but in many ways consistent with a persistent, non-resolving inflammation. In this context, high mobility group box 1 (HMGB1), a prototypical alarmin involved in both sterile and infectious inflammation, has gained interest, particularly since animal studies indicate that HMGB1 promotes neuroinflammation and cognitive impairment.

This thesis investigates aspects of PICS in ICU survivors, including subjective and objective cognitive function, physical performance, and markers of inflammation. Patients were included in two cohorts designed for ICU follow-up studies.

In study I, the association between sepsis and delirium during ICU stay and symptoms of psychological distress (Hospital Anxiety and Depression Scale (HADS) and Post-traumatic Stress Symptoms Scale-10 (PTSS-10)) and self-rated cognitive function (Cognitive Failures Questionnaire (CFQ)) after three months were investigated. There was no significant association between sepsis or delirium at ICU stay and self-rated cognitive function at the three months follow-up. In contrast, there was a strong significant correlation between patients' self-rated cognitive function and symptoms of depression, anxiety, and PTSD.

Studies II-IV investigate a 12 months follow-up cohort of ICU survivors. Patients were examined at the ICU follow-up clinic at three, six, and twelve months after ICU discharge. They underwent formal neuropsychological testing, performed physical tests, and responded to three questionnaires on psychological distress and subjective cognitive function (HADS, PTSS-10, CFQ). Blood samples were collected at the three and six-month follow-up visits.

Study II investigates whether patients´ subjective cognitive function correlates to objectively measured cognitive function. Answers on the CFQ were analyzed together with outcomes on the Cambridge Neuropsychological Test Automated Battery (CANTAB). There was no clinically relevant correlation between subjective and objective cognitive function as measured here. We conclude that subjective cognitive function tests in ICU survivors must be interpreted with caution and that both subjective and objective testing may be necessary to adequately ascertain cognitive function in ICU survivors.

In study III, the association between plasma HMGB1 and objective cognitive function measured in four different cognitive domains (executive function, visual memory, sustained attention, working memory) was investigated. Interestingly, plasma levels of HMGB1 were significantly elevated in the ICU, at discharge, and at the three- and six-months follow-up visits as compared with reference populations. Elevated plasma levels of HMGB1 were associated with reduced sustained attention at the three- and six-month follow-up visits. Based on these findings, further follow-up studies on HMGB1 biology in ICU survivors are warranted to investigate the potential for therapeutic targeting of HMGB1 function in prevention of cognitive impairment in ICU survivors.

In study IV we explore the association between plasma HMGB1 and physical performance at ICU follow-up. We observed no significant association between levels of plasma HMGB1 and the three physical tests performed (i.e., 6-min walk test, timed stands test, handgrip strength test).

In conclusion, this thesis provides new insights on objective and subjective cognitive function, psychological distress, and markers of inflammation in ICU survivors. Future work may build on this knowledge to improve the identification and treatment of at-risk subjects in this vast and growing patient population.

LIST OF SCIENTIFIC PAPERS

I. **The impact of sepsis, delirium, and psychological distress on self-rated cognitive function in ICU survivors-a prospective cohort study.**
Brück E, Schandl A, Bottai M, Sackey P. J Intensive Care. 2018 Jan 8;6:2.

II. **Lack of clinically relevant correlation between subjective and objective cognitive function in ICU survivors: a prospective 12-months follow-up study.**
Brück E, Larsson JW, Lasselin J, Bottai M, Hirvikoski T, Sundman E, Eberhardson M, Sackey P, Olofsson PS. Crit Care. 2019 Jul 12;23(1):253.

III. **Prolonged elevation of plasma HMGB1 is associated with cognitive impairment in intensive care unit survivors.**
Brück E, Lasselin J, Caravaca AS, Gallina AL, Bottai M, Andersson U, Sackey P, Olofsson PS

Submitted

IV. **Plasma HMGB1 levels and physical performance in ICU survivors.**
Brück E, Svensson-Raskh A, Larsson JW, Caravaca AS, Gallina AL, Eberhardson M, Sackey P, Olofsson PS

Manuscript

OTHER PUBLICATIONS NOT INCLUDED IN THE THESIS:

I. **Early psychological screening of intensive care unit survivors: a prospective cohort study.**
Milton A, Brück E, Schandl A, Bottai M, Sackey P. Crit Care. 2017 Nov 9;21(1):273

CONTENTS

LIST OF ABBREVIATIONS

6-MWT	6-min walk test
APACHE	Acute Physiology and Chronic Health Evaluation
ARDS	Acute respiratory distress syndrome
atHMGB1	All-thiol HMGB1
BBB	Blood-brain barrier
CAM-ICU	Confusion Assessment Method for the ICU
CANTAB	Cambridge Neuropsychological Test Automated Battery
CFQ	Cognitive Failures Questionnaire
ChAT	Choline acetyltransferase
CI	Confidence interval
CLP	Cecal ligation and puncture
CLR	C-type lectin receptors
CNS	Central nervous system
DAMP	Damage-associated molecular patterns
dsHMGB1	Disulfide HMGB1
ECMO	Extracorporeal membrane oxygenation
ELISA	Enzyme-linked immunosorbent assay
HADS	Hospital Anxiety and Depression Scale
HMGB1	High mobility group box 1
HST	Handgrip-strength test
ICU	Intensive care unit
IDO	Indoleamine 2,3-dioxygenase
IL	Interleukin
IQCODE	Short Informant Questionnaire on Cognitive Decline in the Elderly
IQR	Interquartile range
JAK	Janus kinase
LPC	Lysophosphatidylcholine
LPS	Lipopolysaccharide
MD	Mean differences
NLR	NOD-like receptors
NMDA-rec	*N*-methyl-D-aspartate receptor
oxHMGB1	sulfonyl HMGB1
PAMP	Pathogen-associated molecular patterns
PICS	Post-intensive care syndrome

PRM	Pattern recognition memory
PRR	Pattern recognition receptors
PTSD	Post-traumatic stress disorder
PTSS-10	Post-traumatic Stress Symptoms Scale-10
RAGE	Receptor for advanced glycation end products
RLR	RIG-I like receptors
RVP	Rapid visual information processing
SAPS	Simplified Acute Physiology Score
SOC	Stockings of Cambridge
SOFA	Sequential Organ Failure Assessment
SSP	Spatial span
STAT	Signal transducer and activator of transcription
TLR	Toll-like receptors
TNF- α	Tumor necrosis factor-α
TST	Timed stands test
α7nAChR	α7 nicotinic acetylcholine receptor subunit

1 INTRODUCTION

1.1 The history of intensive care

In 1952, the polio epidemic ravaged Denmark. Professor Lassen, chief physician of the Blegdam Hospital, watched his patients die from respiratory failure due to paralysis from poliomyelitis. At that time, the hospital had very limited resources in terms or respirators, and Professor Lassen stood powerless in his urge to provide treatment for patients that succumbed to polio. He then contacted Bjørn Ibsen, an anesthetist trained in the U.S, with the hope that his newly gained knowledge in modern anesthesia would come to help (1). Ibsen came to Blegdam Hospital in late August 1952, where, at that time, a 12-year-old girl with severe polio was admitted. In a theater full of colleagues, the young patient was tracheotomized under local anesthesia, and Ibsen could, after general sedation, successfully deliver positive pressure ventilation manually (2). Positive pressure ventilation was new in the field of anesthesia, and when Ibsen's method was applied to all polio patients, mortality rates quickly dropped from approximately 85 % to 30 %. Medical students were recruited for the management of hand ventilation, and designated wards were built to provide around-the-clock medical care (Figure 1). This was the beginning of a new era; the first intensive care unit (ICU) was established.

Figure 1: An 8-year-old girl being manually ventilated through tracheostomy in 1952. *(3)*

2 BACKGROUND

2.1 Post-intensive care syndrome

Several million patients are cared for in ICUs in Europe every year, due to life-threatening illness or injury (4). As the survival rates after intensive care are consistently increasing (5) it has become more recognized that ICU patients suffer from debilitating sequels in the years to come after primary survival. These problems include cognitive impairment, post-traumatic stress disorder (PTSD), depression, functional disabilities, and reduced quality of life in the months to years after ICU discharge (6-11). At a stakeholder conference in North America in 2010, the ICU community came to consensus on these emerging symptoms to improve long-term outcomes in ICU survivors, and the term post-intensive care syndrome (PICS) was introduced (12). PICS is defined as new onset or worsening of psychological, physical, or cognitive impairments that persist past the acute care and hospitalization. The prevalence of PICS varies, but 25-50 % of patients suffer from symptoms from some component of PICS after ICU discharge (8, 13-16). In the last decade, PICS has become recognized as a major socio-economic burden, as the consequences that PICS comprises impede patients' return to the level of function and employment they had before onset of illness (17, 18).

2.1.1 Mental impairment in PICS

Facing death or being severely ill greatly impacts the psychological well-being of ICU survivors, as adverse experiences from the ICU require coping with the new perspectives and life-conditions that most likely will occur. The stress and physiological changes that take place during ICU stay will affect the recovery even of patients with good family networks and high coping ability. Up to 20 % of ICU survivors suffer from depression, anxiety, and PTSD, and risk factors include history of psychological problems, female gender, and education level (9, 10, 19). The prevalence of depression is also significantly higher in ICU survivors than in the general population (8 %) (20). Both intervention and rehabilitation have proven challenging in this patient group (16, 21, 22). Nevertheless, early screening to identify patients at risk and the use of ICU diaries to help with coping has emerged as possible helpful interventions (23, 24). PICS symptoms do, however, often co-occur, and understanding the relationship between the different domains of PICS is one of many challenges the scientific and clinical ICU community faces (16).

2.1.2 Physical impairment in PICS

Physical disabilities after critical illness include loss of muscle mass, muscular weakness, reduced lung capacity, nerve dysfunction, and fatigue. The co-occurrence of these symptoms is called "ICU-acquired weakness", and is present in almost half of patients with sepsis or multi-organ failure (25). A diagnose of ICU-acquired weakness includes critical illness myopathy, polyneuropathy, and neuromyopathy assessed through electrophysiological tests, and the neuropathy is characterized by axonal degeneration without demyelination (26). ICU-acquired weakness is associated with prolonged ICU and hospital stay due to reduced functional capacity, and therefore difficulties in weaning patients from mechanical ventilation (27). Apart from functional disabilities, ICU-acquired weakness has also been found to be an independent predictor of hospital mortality (28). The physical disability in ICU-acquired weakness may persist for years after ICU discharge, and a prospective follow-up study of ARDS patients showed reduced physical capacity for up to five years (14, 29). These impairments, together with the other PICS symptoms, affect the trajectory of recovery and have an impact on both patients' daily function as well as their ability to return to their previous employment (18, 30).

The pathophysiology behind ICU-acquired weakness is still unclear, but multiple factors have been suggested (31). These include microvascular ischemia due to disturbed microcirculation, which results in neuronal and mitochondrial injury and axonal degeneration. Additionally, the catabolic state and immobility, which occur early in ICU patients, can lead to skeletal muscle wasting and loss of muscle mass (31). Inflammation, particularly impaired resolution of inflammation, has furthermore been proposed as playing an important role in the pathophysiological mechanism behind the development of physical impairment in ICU survivors (32).

2.1.3 Cognitive impairment in PICS

The cognitive functions most commonly affected after critical illness are memory, attention, visual-spatial ability, and executive function (33-35). Cognitive impairment after intensive care is reported in up to 2/3 of survivors, but the prevalence varies depending on follow-up time and chosen cognitive tests (13). As with the other impairments in PICS, the pathogenesis of cognitive impairment after critical illness is still unclear. In part it may be an accelerated neurodegenerative progression in susceptible patients (e.g., ICU patients are older, might have pre-existing cognitive dysfunction and reduced cognitive reserve), or newly obtained brain injury due to insults from being critically ill (e.g., hypotension, hypoxia, anemia, systemic inflammation, sepsis, pharmacological treatment, renal failure, and liver failure) (36-38) (Figure 2).

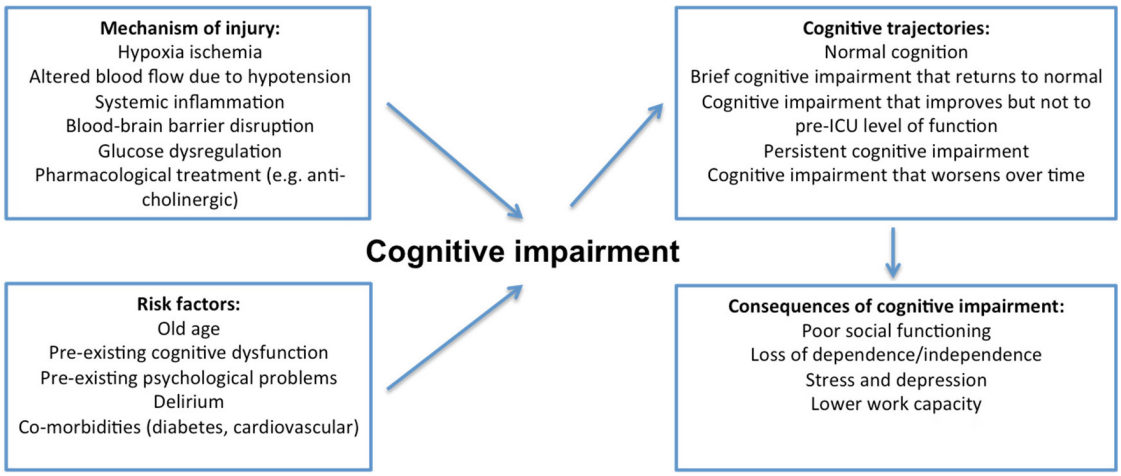

Figure 2: Mechanisms and consequences of post-ICU cognitive impairment. *Modified from (39).*

As of today, length of delirium is the only proven independent risk factor for worsened cognitive impairment (6, 7). Other factors previously thought to impact patient outcome include the use of benzodiazepines, duration of mechanical ventilation or hypo- and hyperglycemia, have, however, shown no, or only weak associations (7, 40, 41). Additionally, although sepsis in a large retrospective study have shown association with the development of cognitive dysfunction (36), traditional indicators of disease severity often fail to predict the future severity of cognitive impairment (39). As such, the connection between severity of illness and severity of impairment remains elusive.

In addition, assessment of cognitive function after critical illness is challenging, since there is a lack of consensus on what, how, and when to test. Most prospective studies on critically ill patients have not used formal neuropsychological tests. Instead, the results are often based on interviews in person, or even by proxy, and on tests that were developed to identify Alzheimer's disease in an elderly population (42). This methodology is not optimal to detect mild cognitive impairment, often prevalent in ICU survivors. This is important, since even mild cognitive impairment may have significant effects on recovery, particularly in working individuals. Furthermore, self-rated screening tools of cognitive function are often interpreted as objective measures of cognition (43), a problematic inference given patients' inability to self-assess (44). Conversely, formal neuropsychological testing might not reliably indicate problems in everyday life, and both subjective and objective measurements may be of equal importance to the patient (45).

2.2 Inflammation, sepsis, and delirium

The immune system comprises the innate and adaptive systems, which together act as our body´s defense mechanism against microbial threats and injury (46). Infectious products from bacteria (exogenous) and non-infectious products from

sterile injury (endogenous) activate the innate immune systems through pattern recognition receptors (PRR) (47). The PRRs recognize exogenous products such as lipopolysaccharide (LPS) from Gram-negative bacteria and endogenous products such as mitochondrial DNA released from injury. Both microbial products, called pathogen-associated molecular patterns (PAMP), and non-microbial products, called damage-associated molecular patterns (DAMP), bind to some extent to the same PRRs (48). Four families of PRRs are presently defined: Toll-like receptors (TLR), NOD-like receptors (NLR), C-type lectin receptors (CLR), and RIG-I like receptors (RLR) (49). Most of the PRRs, when activated, increases the production of pro-inflammatory cytokines, leading to a systemic inflammatory response (49). This systemic inflammatory response due to exogenous products is described clinically as sepsis. However, both sterile and infectious products may activate similar immune responses, whether the criteria for a sepsis diagnosis are met or not (50, 51).

2.2.1 Sepsis

Sepsis is a life-threatening condition, occurring in more than one-third of ICU patients at some point (52). The definition of sepsis has changed over the years with the present-day Sepsis-3 criteria (53) being more rigid than the earlier criteria developed in 1991 (54). Sepsis survivors have a high short- and long-term mortality rate and sepsis is associated with a significant burden of morbidity (55, 56). This includes multiple organ failure, critical illness myopathy, and acute confusion (delirium). Intensive efforts over several decades to develop effective treatment of the acute episode have had limited success. The reason for this increased mortality and morbidity after primary survival is unclear, but one hypothesis is that post-septic patients suffer from a phenomenon termed “nonresolving inflammation” (57).

In critical illness, the systemic inflammatory response can develop into multiple organ dysfunction, including brain dysfunction (58). This brain dysfunction is not due to infection of the central nervous system (CNS) but instead caused by mediators released during sepsis (58). The severity of inflammation impacts its influence on cognitive function. In mild cases, systemic inflammation can cause minor cognitive changes, and in more severe cases, it may present itself clinically as delirium (58, 59).

2.2.2 Delirium

Delirium is an alteration and fluctuation, in attention and cognition that occurs in up to 30-80 % of ICU patients (60-63). The occurrence and duration of delirium have been independently associated with prolonged hospitalization and increased mortality and morbidity (60, 61, 64). Some studies have reported an association between sepsis, delirium, and subsequent decline in cognitive function, but it is currently unclear whether these clinical symptoms can accurately predict later cognitive impairment (6, 7, 36, 40).

Regarding sepsis-associated delirium, two possible mechanisms have been suggested: sepsis-induced ischemia that leads to inadequate cerebral perfusion and inflammatory mediators leading to mitochondrial dysfunction; and microglial and endothelial activation, which results in impaired microcirculation, apoptosis and the release of neurotoxic substances (65). The endothelial activation plays a central part in reduction of the blood-brain barrier (BBB) integrity. Disruption of the BBB during sepsis can enable pro-inflammatory factors to enter the CNS, including PAMPs and DAMPs. These initiate neuroinflammation through microglia activation, which is involved in the development of encephalopathy (66). The elevated levels of cytokines in the brain that occur during sepsis can induce apoptosis of cholinergic neurons. Levels of the neurotransmitter acetylcholine may thus decrease and lead to cholinergic hypofunction, which may augment cognitive decline (67).

2.3 Inflammation and cognitive impairment

Neuroinflammation occurs when the innate immune system is activated within the CNS, due to inflammatory signals such as pathogen infection, injury, or trauma. Neuroinflammation triggers not only cognitive decline but also behavioral changes, described as sickness behavior (68). Sickness behavior includes symptoms of lethargy, anorexia, depression, social withdrawal, hyperalgesia, and cognitive disturbance (69). Increased levels of cytokines in the CNS have multiple effects, including activation of the enzyme indoleamine 2,3-dioxygenase (IDO) involved in the Kynurenine pathway (70). The Kynurenine pathway is the central route for tryptophan metabolism. IDO promotes the synthesis of Kynurenine, resulting in reduced bioavailability of tryptophan, which regulates the rate of serotonin synthesis in the brain and has significant psychological consequences.

ICU-survivors suffer from PICS symptoms such as depression and reduced health-related quality of life. This may not just be the aftermath of being critically ill. Instead, it might be triggered by acute inflammation and further aggravated by the non-resolving inflammation. Sickness behavior has also been suggested as an important factor contributing to worse self-rated health (69).

The pathophysiology of cognitive dysfunction after critical illness is currently not known. Murine studies of experimental sepsis show a link between elevated levels of the DAMP high mobility group box 1 (HMGB1) and cognitive impairment in murine sepsis survivors. Interestingly, HMGB1 in plasma was elevated for eight weeks in mice surviving cecal ligation and puncture (CLP), and this elevation was associated with cognitive dysfunction (71). The mice showed problems in learning and memory weeks after sepsis, along with reduced hippocampal dendrite density and loss of synaptic plasticity (71). Administration of a monoclonal antibody targeting HMGB1 seven days after CLP abolished the cognitive impairment. In addition, injection of HMGB1 in healthy mice induced similar pathological changes as those found in CLP mice (71) (Figure 3).

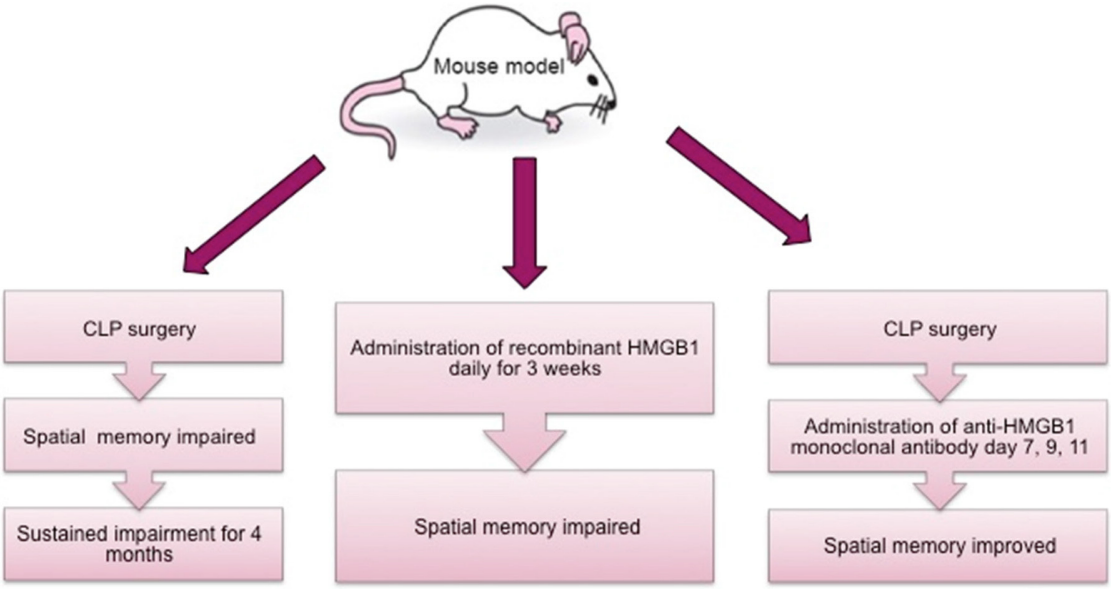

Figure 3: Experimental design in the Chavan et al. study from 2012. *In an experimental model of sepsis, i.e. cecal ligation and puncture (CLP), in which the cecum is ligated below the ileocecal valve and then punctured in anesthetized mice, HMGB1 was associated with cognitive impairment.*

2.4 HMGB1

HMGB1, discovered in 1973 as a chromatin-binding molecule, is an evolutionarily highly conserved nuclear protein named for its high electrophoretic mobility (72). It is an approximately 25 kDa protein composed of 214 amino acids arranged in two DNA-binding domains, the A box and B box, together with a C-terminal tail (73). HMGB1 is actively secreted by immune cells in response to pathogenic products and injury, or passively released by apoptotic and necrotic cells (Figure 4). Its role as a prototypical DAMP or alarmin was discovered in 1999, when it was found to be elevated in mice during endotoxemia (74).

In the nucleus, HMGB1's primary function is to maintain genome stability and regulate gene transcription (72). However, when the nuclear localization sequence (NLS) of HMGB1 becomes hyperacetylated through posttranslational modification, it will translocate to the cytosol (75). Actively released HMGB1 is thus hyperacetylated, whereas HMGB1 passively released from dying or injured cells is not. The active secretion from the cytosol to the extracellular milieu is not entirely understood, as HMGB1 lack a secretory leading peptide and is therefore not transported through the classical endoplasmic reticulum-Golgi pathway. Instead, HMGB1 seems to be accumulated in secretory lysosomes from where it can be released into the extracellular milieu in response to inflammatory signals (76). This system is similar to how other pro-inflammatory cytokines such as IL-1β are released, which is rapidly secreted from lysosomes in response to extracellular ATP that is high in inflammatory conditions (78). However, the exocytosis of HMGB1 is different as it is induced by lysophosphatidylcholine (LPC), a lipid that is produced by immune cells, usually at the site of infection (76).

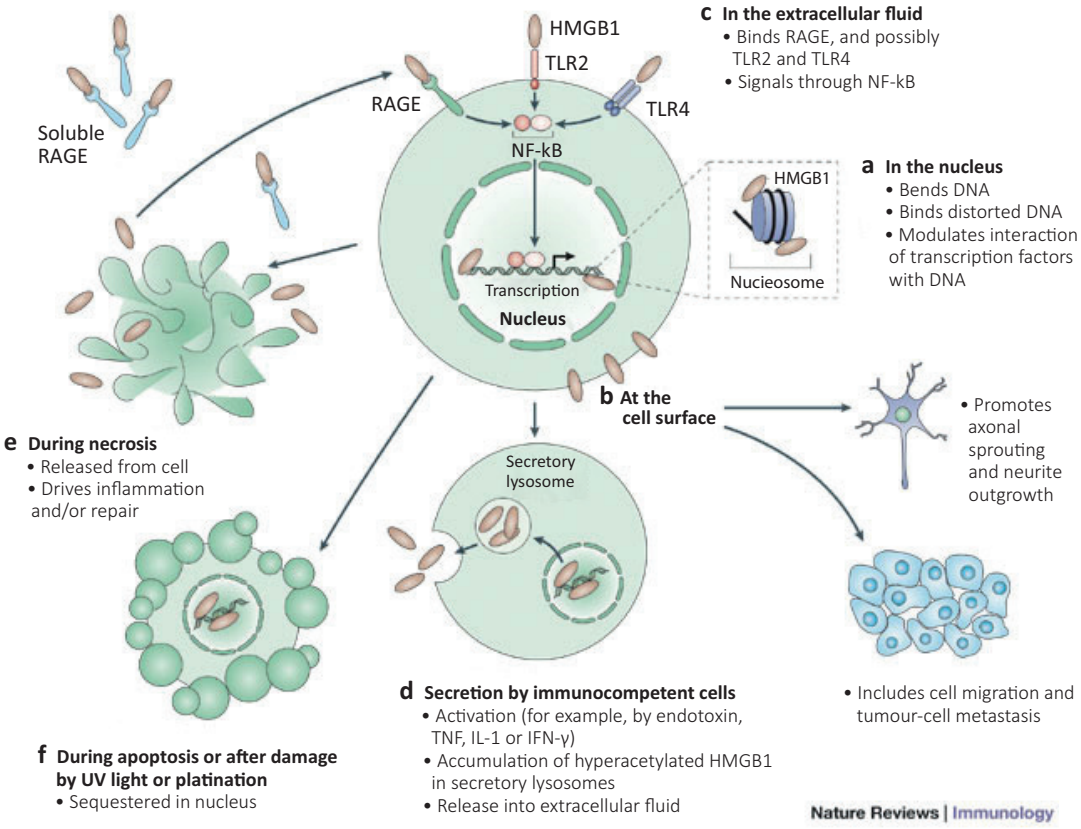

Figure 4: *a) HMGB1 bends DNA in the nucleus and modulates interaction of transcription factors with DNA. b) HMGB1 induces cell migration and promotes axonal growth at the cell surface. c) HMGB1 binds to RAGE, TLR 2 and 4 and signals through NF-kB d) HMGB1 is actively secreted by immunocompetent cells e) HMGB1 is passively released during necrosis f) HMGB1 sequestered in the nucleus after apoptosis (77). Reprinted with permission from publisher.*

In the extracellular milieu, HMGB1's function to regulate inflammation is dependent on the redox state of cysteine residues at position 23, 45, and 106 (79). This redox state is variable and to a great extent depending on the redox balance in the intra-and extracellular milieu. When the three cysteine residues are in their fully reduced form with thiol residues, the complex is inactive, as is the state of nuclear HMGB1. Oxidation of HMGB1 creates a disulfide bond between Cys 23 and Cys 45, causing it to switch into a potent pro-inflammatory cytokine (80). When fully oxidized, HMGB1 is in its irreversible sulfonyl form, which has no known immunologic function (80). Receptors that mediate HMGB1 signaling include receptor for advanced glycation end products (RAGE), TLR 2, -4, and -9, among others, where TLR-4 is the primary receptor for macrophage activation and cytokine release (81). The redox state of HMGB1 regulates its ability to bind to the different receptors (Figure 5). TLR-4 activates the NF-κB pathway, through MyD88, an intracellular adaptor protein, that can activate the transcription factor NF-κB (82). NF-κB activation promotes the transcription of pro-inflammatory cytokines and chemokines (chemoattractant cytokines) in several cell types, including

macrophages, mast cells, and endothelial cells (82). Central cytokines in inflammation involve tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, which are usually produced locally, at the site of infection. However, cytokines, DAMPs, and PAMPs may enter the circulation and induce a systemic inflammatory response, which often occurs in infection or inflammatory diseases (83).

HMGB1, in the extracellular milieu, is prone to synergize and form complex with several other pro-inflammatory molecules, including DNA, RNA, histones, nucleosomes, LPS, CXCL-12, IL-1α, and IL-1β (84). This molecular collaboration is an important mechanism for HMGB1-mediated inflammation (85-87). Extracellular HMGB1-partner molecule complexes bind to RAGE on cell surfaces and become endocytosed to the endolysosomal compartment. HMGB1 then acts as a detergent under the acidic conditions in lysosomes, where HMGB1 disrupts the lysosomal membrane and enables its partner molecules to avoid degradation and to escape to the cytosol to activate reciprocal intracellular receptors (84, 85). One essential example of this mechanism is that LPS-toxicity in sepsis involves HMGB1-assisted transport (85). When LPS enters the cytosol through HMGB1-RAGE-mediated entrance and avoids degradation, it will intracellularly activate Caspase-11, which in turn cleaves Gasdermin D into peptides that create pores in the cytoplasmic membrane (88, 89) (Figure 6). This process is called pyroptosis ("pyro" from greek "fire") and is a highly inflammatory form of programmed cell death (90). One major source of HMGB1-release in experimental sepsis is hepatocytes (85). Experimentally, deletion of HMGB1 in hepatocytes, neutralizing extracellular HMGB1, loss of caspase-11, or deficiency in Gasdermin-D, all independently improved survival in mouse models of sepsis (85, 91).

In summary, HMGB1´s function depends on its location, molecular binding partners, and redox state (92).

Activation of the systemic inflammatory response is also regulated through the brain stem and the neural circuits of the vagus nerve (93). Sensory nerve fibers report on cytokine levels and inflammation in the periphery, and signals to the brain via the afferent vagus nerve. The brain stem processes this information, which activates the efferent vagus nerve to inhibit the release of pro-inflammatory cytokines through the "inflammatory reflex arc" (93). Activation of this anti-inflammatory reflex results in the activation of adrenergic neurons in the spleen that promotes splenic T cells to release acetylcholine (94, 95). Acetylcholine binds to α7 nicotinic acetylcholine receptor subunit (α7nAChR) on monocytes/macrophages and inhibits the release of pro-inflammatory cytokines (96, 97) (Figure 7). Interestingly, this neural circuit also regulates inflammasome activation and serum HMGB1 levels (98, 99). This is particularly interesting in light of the potential therapeutic use of selective nerve stimulation in treatment of excessive inflammation (100).

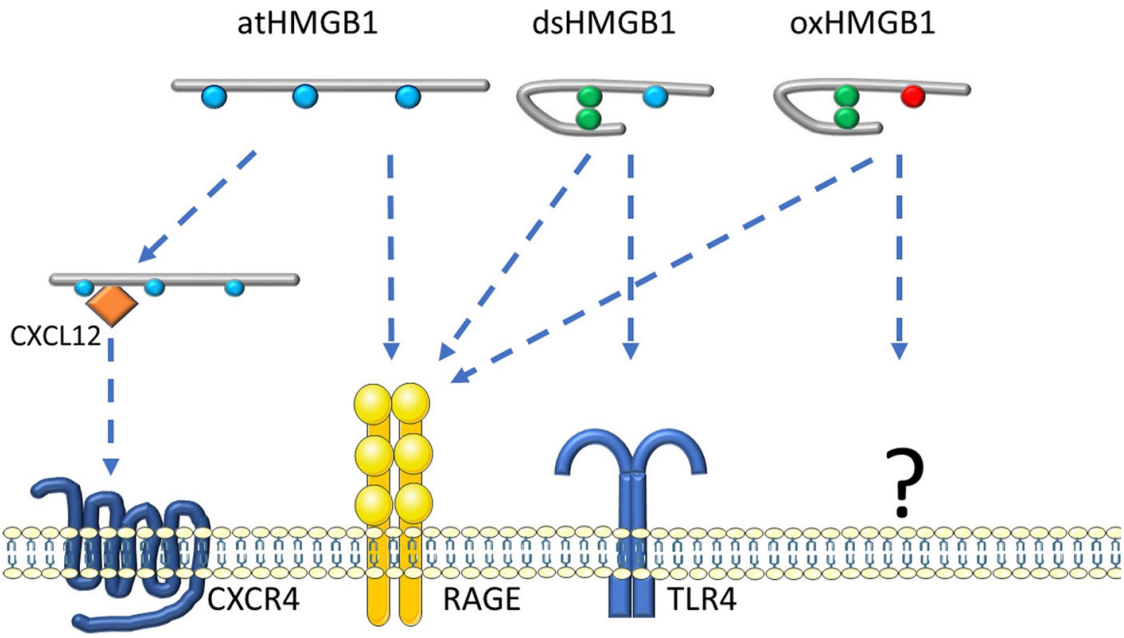

Figure 5: The different redox forms of HMGB1 regulate receptor binding. *All-thiol HMGB1 (atHMGB1) forms a complex with CXCL12 that signals via CXCR4 and have chemotactic properties. Disulfide HMGB1 (dsHMGB1) signals via TLR4 with pro-inflammatory effects. AtHMGB1, dsHMGB1 and sulfonyl HMGB1 (oxHMGB1) all bind to RAGE (84). Reprinted with permission.*

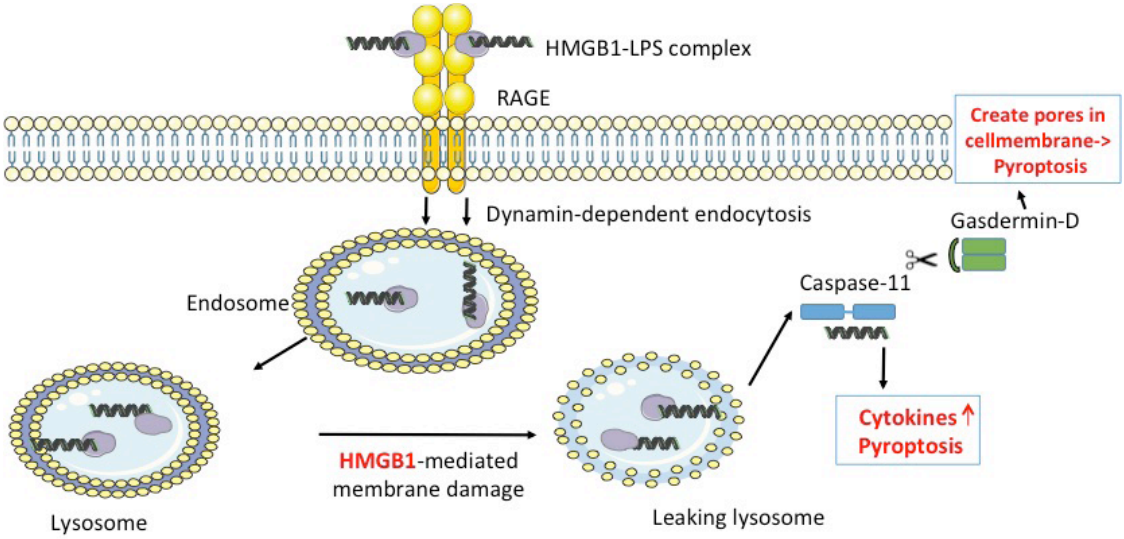

Figure 6: Extracellular HMGB1-partner molecule complexes bind to cell surface-expressed RAGE and become endocytosed to the endolysosomal compartment. *HMGB1 then acts as a detergent under the acidic conditions in lysosomes, where HMGB1 disrupts the lysosomal membrane, enabling LPS to avoid degradation and to escape to the cytosol to activate Caspase-11. Caspase-11 then cleaves Gasdermin-D into peptides that create pores in the cell membrane, and pyroptosis occurs. (Copyright: Ulf Andersson)*

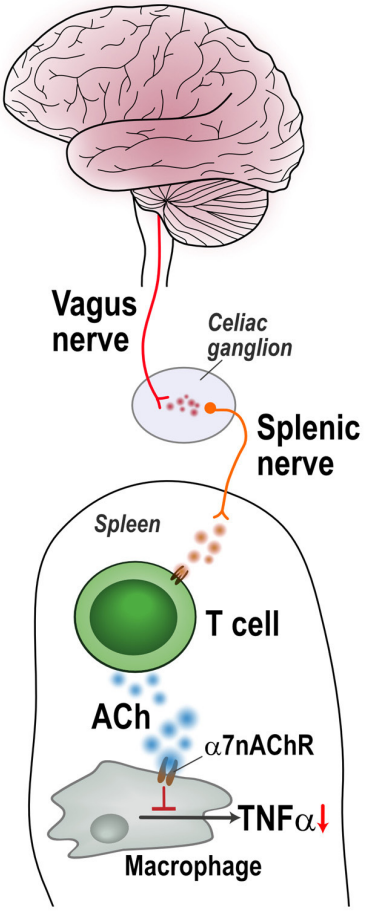

Figure 7: Signals in efferent vagal neurons functionally activate adrenergic nerve terminals in the spleen to release norepinephrine, which promotes acetylcholine release from choline acetyltransferase (ChAT)+ T cells. *Acetylcholine binds to α7nAChR on macrophages, and suppresses synthesis and release of pro-inflammatory cytokines, e.g. TNF-α, IL-1, IL-18 and others. (Copyright: Laura Tarnawski)*

2.4.1 HMGB1 in neuroinflammation

HMGB1 plays a central role in promoting neuroinflammation through activation of microglia, the predominant innate immune cell in the brain (101, 102). Microglia express TLRs, in particular TLR4, one of HMGB1s main receptors (103), and activation of microglia with elevated systemic HMGB1 levels have in experimental models correlated with cognitive dysfunction (104). HMGB1 is also capable of disrupting the blood-brain barrier and thus enables entry of other pro-inflammatory mediators, further aggravating the inflammation (105, 106). In addition to TLR-4 and RAGE, the *N*-methyl-D-aspartate receptor (NMDA-rec) is important in HMGB1 mediated neuroinflammation. NMDA-rec are known to regulate neurotransmission, but also synaptic plasticity, learning, memory, and cognition (107). HMGB1 have pro-excitatory effects on the NMDA-rec in the hippocampus through increased phosphorylation of the receptor and, therefore, calcium channel conductance (108).

HMGB1´s function in neuroinflammation has been extensively studied in different disease models, including traumatic brain injury (109), ischemic brain lesions (110), epilepsy (111), Alzheimer´s disease (112), stress (67), and depression (113).

Furthermore, HMGB1 has been shown to cause hippocampal inflammation and cognitive impairment in both experimental sepsis and major sterile trauma in mice (71, 102, 114-117). Collectively, these observations support a functional role of HMGB1 in the development of neuroinflammation and cognitive impairment, both in sepsis and sterile inflammation.

As mentioned earlier, many studies targeting a number of pro-inflammatory mediators have shown great promise in attenuating sepsis in murine models. However, clinical trials built on these experimental discoveries have ultimately failed (118-120), and ever since pro-inflammatory cytokines were found to be elevated in patients with severe infection (121), the focus has been on finding therapy targeting this response. A vital difference between HMGB1 and other pro-inflammatory cytokines is that it is a late mediator of inflammation (74). In contrast to other cytokines released in sepsis, such as TNF-α, IL-1, and IL-6, which return back to baseline levels within hours after sepsis onset, HMGB1 may stay elevated for weeks after an acute event (122-124). This difference is important, as it gives a wider therapeutic time window to administer treatment. Administration of antagonists of HMGB1 to mice, e.g. monoclonal antibodies or recombinant HMGB1 box A protein, up to 24 h *after* disease onset significantly improved survival (125). Moreover, monoclonal anti-HMGB1 antibodies administrated to mice 6, 9, and 11 days after CLP, as described in the Chavan *et al*. study, significantly improved memory function (71). This suggests a possibility of great clinical importance and a promising new potential molecular target for therapy in severe systemic inflammation (80).

2.4.2 HMGB1 in muscle dysfunction

Cytokines and other mediators of inflammation play essential roles in the regulation of skeletal muscle through their effects on metabolism, protein turnover, and cell differentiation (126). A key feature in ICU-acquired weakness is the loss of myosin filaments and the disruption of the myofilament organization of the tissue (127). Activation of the NF-κB pathway will increase not only the production of pro-inflammatory cytokines, but also the expression of ubiquitin–proteasomes that are involved in muscle-specific atrophy (128), and as mentioned earlier, HMGB1 activates the NF-κB pathway. Furthermore, experimental data indicate that HMGB1 impairs muscle Ca^{2+} homeostasis by HMGB1 signaling through TLR4 (129), and that RAGE, the other main receptor of HMGB1 has found to be involved in skeletal muscle turnover (130). However, little is known about the long-term course of inflammation and its association with physical impairment in ICU survivors.

Considerable challenges lay ahead in both the field of PICS and HMGB1 biology. The complexity and heterogeneity of the ICU population, together with the lack of baseline data in this patient group due to acute admission, thwarts progress. The studies presented in this thesis will, hopefully, shed some small light on the mechanisms behind the impairments in post-intensive care syndrome.

3 AIMS

Study I: (a) To examine the relationship between sepsis, ICU delirium, and later self-rated cognitive function in ICU survivors.

(b) To investigate the association between depression, anxiety, PTSD, and self-rated cognitive function after ICU stay.

Study II: To investigate if subjective cognitive function correlate to objective cognitive function as measured by Cambridge Neuropsychological Test Automated Battery (CANTAB) in ICU survivors.

Study III: To investigate plasma HMGB1 levels in ICU survivors, and if elevated plasma HMGB1 levels are associated with cognitive dysfunction as measured by neuropsychological tests (CANTAB).

Study IV: To examine if plasma HMGB1 levels are associated with physical performance in ICU survivors.

4 METHODS

4.1 Ethical considerations

The Stockholm regional ethics committee approved studies I-IV, which were conducted in accordance with the Helsinki declaration (131), and good clinical practice. All patients included in the studies gave written informed consent. In order to protect patients' autonomy, we emphasized that participation was entirely voluntary, and patients had the opportunity to cancel their participation at any time. Blood samples were taken through existing vascular access at the hospital, and at two time-points during follow-up. The risk of inflicting harm on the study patients was considered minimal. In studies II-IV, the follow-up offered was more thorough compared to standard care, and patients may have benefitted from this.

4.2 Description of studies

Detailed descriptions of the method can be found in the method section in the respective papers.

4.2.1 Study I

In this prospective cohort study, data were collected from January 2012 until February 2013. Part of the patients was included in an international multicenter study (PRE-DELIRIC) (132). Eligible for inclusion were patients staying longer than 24 hours at the ICU. Patients who were mentally impaired, had severe auditory or visual disorders, suffered from aphasia, were unable to understand Swedish, or were transferred to another ICU were excluded. Patients that were sedated during their entire ICU stay and had a Richmond Agitation and Sedation Scale of minus four or less were also excluded.

Patients were screened for delirium daily at the ICU using the Confusion Assessment Method for the ICU (CAM-ICU) (133), and their sepsis status was noted. In this study, we diagnosed patients with the standard definitions used at the time of patient inclusion (Table 1). As sepsis without any organ dysfunction is commonly found in the ICU, we chose patients with severe sepsis and septic shock to be defined as one group, in order to distinguish patients more affected by their infection.

Study participants received three questionnaires by postal mail; CFQ (measures self-rated cognitive function) (134), HADS (measures anxiety and depression) (135), PTSS-10 (measures symptoms of post-traumatic stress) (136), three months after their ICU discharge. Independent variables of the study were sepsis or delirium at the ICU, and depression, anxiety, and PTSD at the follow-up. The outcome variable was patients' self-rated cognitive function measured by the CFQ at follow-up.

Clinical definitions of sepsis	
Systemic inflammatory response syndrome (SIRS)	Two or more of the following • Temperature >38°C or <36°C • Heart rate >90/min • Respiratory rate >20/min • White blood cell count >12x10^9 or <4x10^9
Sepsis	SIRS and evidence of infection
Severe sepsis	At least one sign of sepsis associated organ dysfunction, hypoperfusion or hypotension, including lactate acidosis, oliguria, or acute alteration of mental state
Septic chock	Severe sepsis with hypotension (systolic blood pressure <90mmHg or a reduction from baseline >40mmHg) refractory to adequate fluid resuscitations or need of inotropic drug

Table 1: Clinical definitions of sepsis used at the time of study inclusion.

4.2.2 Study II-IV

Data were prospectively collected from May 2014 to January 2018 for **study II-IV.** Patients staying more than 24 hours and aged 18-70 were included. In order to recognize patients who were cognitively intact before admittance to the ICU, narrow exclusion criteria were used. This included patients with severe auditory or visual disorders, aphasia or inability to understand Swedish, diagnosed with mental impairment or dementia, ongoing alcohol or drug abuse, psychiatric illness and/or psychiatric pharmacological treatment. Further, to identify patients with undiagnosed pre-existing dementia, the Short Informant Questionnaire on Cognitive Decline in the Elderly (IQCODE) (137) was used to screen all patients above 50 years of age, where patients with a high score (i.e. >3) were excluded. Additionally, patients receiving extracorporeal membrane oxygenation (ECMO), patients diagnosed with meningitis, patients with structural brain injury, patients in palliative care, or patients unlikely to survive to follow-up (3 months) were also excluded. Patients with an ICU stay of less than 48 hours were excluded as a short ICU stay may have less of an impact on the outcome. Patients transferred to another ICU or patients residing outside Stockholm were also excluded, as follow-up would not have been feasible.

Patient characteristics and medical data form ICU stay (i.e. APACHE II, SAPS III, presence of delirium, sepsis status, mechanical ventilation) were obtained from the electronic management data system (Take Care, Clinisoft). In **study III,** we diagnosed patients according to the Sepsis-3 criteria (Table 2) (53, 138).

For **study III,** blood samples were collected during hospital stay, and at three and six months after ICU discharge. For **study IV,** the same measurement was used at the three and six month follow-up. In **study III,** plasma was also obtained from 22 healthy subjects from Sweden and used as a reference group for HMGB1 (139).

Sepsis-3	
Sepsis	Evidence of infection and an increase in SOFA score >2
Septic chock	Sepsis with hypotension that despite fluid resuscitations require inotropic drug to maintain MAP ≥ 65 mmHg, and lactate > 2 mmol/L

Table 2: Criteria for Sepsis-3. *The Sequential Organ Failure Assessment (SOFA) score is calculated from severity of illness in six organ systems; respiratory (PaO_2/FiO_2), hepatic (bilirubin), cardiovascular (blood pressure and vasopressors), coagulation (platelets), central nervous system (Glasgow coma scale), and renal system (creatinine and urinary output)*

Patients came to the follow-up clinic for intensive care at three, six, and twelve months after ICU discharge (Figure 8). They there performed formal neurophysiological testing assessed with the CANTAB (CANTAB® [Cognitive assessment software], Cambridge Cognition (2013)). Four different cognitive tests from the CANTAB battery were chosen to measure executive function (Stockings of Cambridge (SOC)), working memory (Spatial span (SSP)), visual memory (Pattern recognition memory (PRM)) and sustained attention (Rapid visual information processing (RVP)) (Table 3). Patients also performed three physical tests; the 6-min walk test (6-MWT), the handgrip-strength test (HST), and the timed stands test (TST) to measure physical performance. Along with the cognitive and physical tests, they handed in three questionnaires that measure self-rated cognitive function (CFQ), symptoms of anxiety and depression (HADS), and PTSS-10 that measures symptoms of posttraumatic stress.

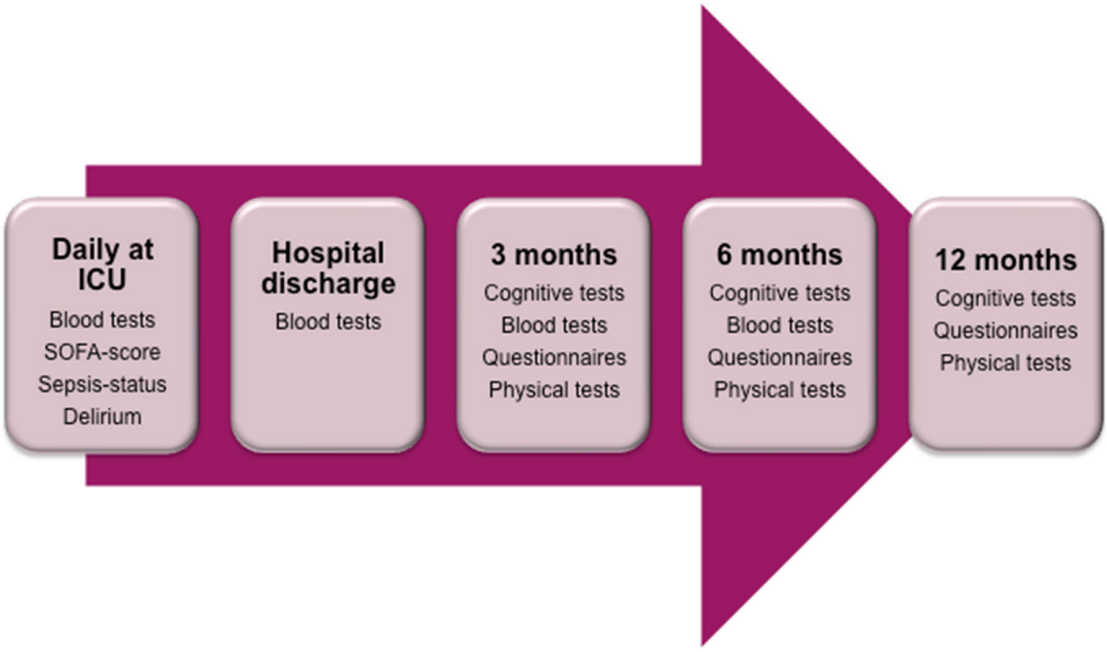

Figure 8: Study design of study II-IV.

In **study II,** was the correlation between the outcome variable CFQ and the variables of the four different CANTAB tests over the 12-month follow-up measured.

The independent variable in **study III** was plasma HMGB1 levels and the outcome variable the score on the four different chosen CANTAB tests (i.e SOC, SSP, PRM, RVP) from the three and six-month follow-up.

In study IV, the independent variable was HMGB1 plasma levels at three and six months and the outcome variable the score on the three physical tests (6-MWT, HST, and TST) at the same time-points.

Test chosen	Area	Function
Pattern recognition memory (PRM)	Temporal	Visual memory
Spatial Span (SSP)	Frontal, Hippocampal	Working memory
Stockings of Cambridge (SOC)	Frontal	Executive functioning
Rapid visual information processing (RVP)	Frontal and parietal	Sustained attention

Table 3: Chosen CANTAB test for neuropsychological testing, which area of the brain that is involved and what function they test.

4.3 Measurement of HMGB1

Blood samples were centrifuged at +4 °C for 10 minutes, at 1000g within 30 min from collection. Plasma was stored at -80 °C for later analyses. Plasma HMGB1 concentrations in ICU survivors and healthy subjects were measured using an enzyme-linked immunosorbent assay (Shino-test HMGB1 ELISA, #ST51011; IBL International, Hamburg, Germany) according to the manufacturer instructions.

4.4 Statistics

Numeric variables were summarized with medians and interquartile ranges and absolute and relative frequencies were reported for categorical variables. Questionnaire data were treated as ordinal.

In **study I,** the association between ICU delirium and severe sepsis/septic shock was analyzed using logistic regression with crude and adjusted (adjusted for; APACHE II score, diabetes mellitus, substance abuse, history of psychological problems) odds ratio calculated. Generalized estimating equations was used in **study I** assessing the association between severe sepsis/septic shock, ICU delirium, and later self-rated cognitive function (CFQ) and presented as mean differences with 95 % CI. The possible combined effect of sepsis and delirium on CFQ scores in **study I** was analyzed using Kruskal-Wallis one-way analysis of variance.

Spearman's rank correlation was used in **study I and II** when analyzing the correlation between psychological distress (HADS and PTSS-10) and self-rated cognitive function (CFQ), and in **study II** analyzing correlation between subjective (CFQ) and objective (CANTAB) cognitive function, rendering the Spearman correlation coefficient known as rho or r.

Linear mixed models were used in **study II-IV** with a patient-specific random intercept included in all models to account for intra-patient correlations. Fixed factors included in the model of **study III** entails the logarithm of HMGB1, age, gender, education level, and the effect of time. In **study IV** the logarithm of HMGB1, age and gender were includes as fixed factors. HMGB1 was log-transformed using the natural logarithm to handle skewness. Pairwise deletion was performed to handle missing data.

The sign test was used in **study III** to compare the z-score median of our cohort compared to British normative data provided by the CANTAB company.

Stata versions 14 and 15 (StataCorp, College Station, TX, USA) and GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA) were used for statistical analyses, and p-values <0.05 were considered significant.

5 RESULTS

5.1 Study I

A total of 754 patients were screened, 514 excluded, and 20 patients died before follow-up, resulting in 216 patients receiving the questionnaires three months after ICU discharge. Of those, 60 % (N=125) of the patients responded to all three questionnaires.

Patients with severe sepsis or septic shock at the ICU were more prone to develop ICU delirium with a crude OR of 3.7 (95% CI, 1.7–8.1), and adjusted OR of 2.9 (95% CI, 1.2–7.2) (adjusted for APACHE II score, diabetes mellitus, history substance abuse, or history of psychological problems) (Figure 9).

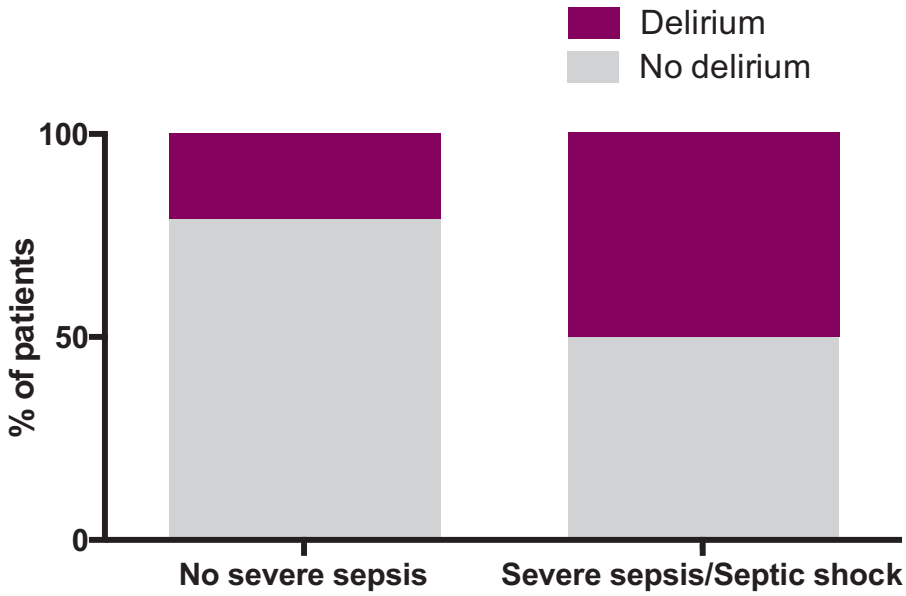

Figure 9: The incidence of delirium was significantly higher in patients with severe sepsis/ septic shock with an adjusted odds ratio of 3.7 and 95% confidence interval of 1.7-8.1.

There was no significant association between CFQ scores for patients with delirium or severe sepsis/septic shock or those without (Table 4). We also investigated the possible interaction of sepsis and delirium on CFQ scores without significant findings (Figure 10)

	Cognitive failure questionnaire score	
	Crude model MD (95% CI)	Adjusted model* MD (95% CI)
No severe sepsis/septic shock (reference)	0	0
Severe sepsis/septic shock	-1.73 (-5.69 to 2.23)	-2.76 (-6.65 to 1.12)
Delirium	1.44 (-2.69 to 5.58)	0.61 (-3.32 to 4.54)

Table 4: Mean differences (MD) in CFQ score and 95% confidence intervals. *No severe sepsis/septic shock was set as a reference. There was no significant difference between groups using generalized estimations equations.*

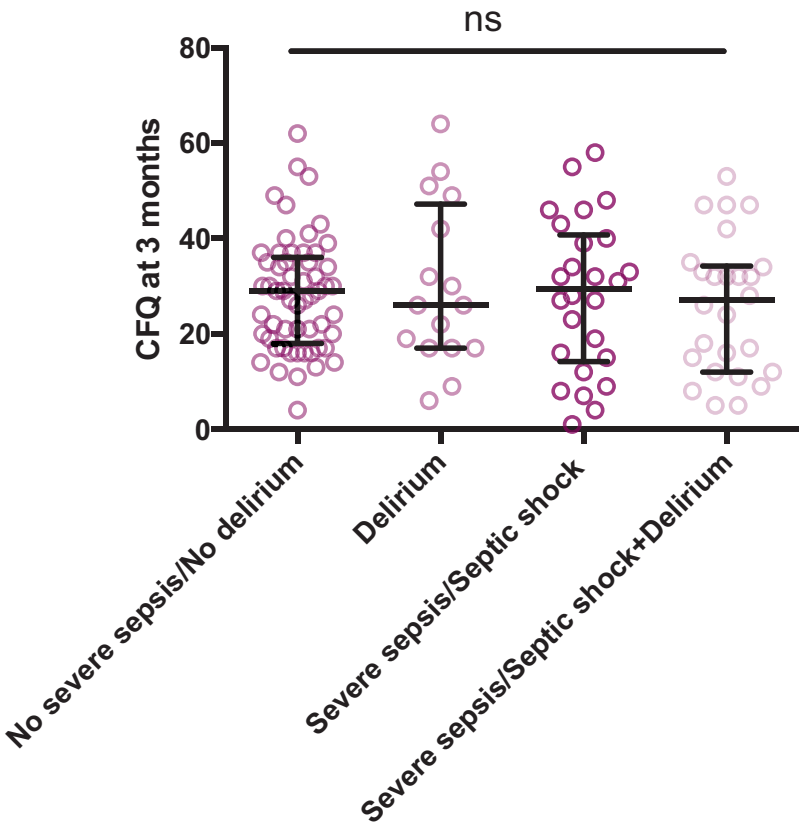

Figure 10: Patients´ individual CFQ score at three months is plotted divided in four groups based on the presence of delirium or severe sepsis/septic shock at ICU stay. *Lines represent median and whiskers the interquartile range. ns = not significant.*

Scores on PTSS-10 and HADS both significantly correlated with the CFQ scores, i.e. patients with more symptoms of depression, anxiety, and PTSD had worse self-rated cognitive function at three months after ICU discharge ($r = 0.44\text{-}0.53$ $p < 0.001$).

5.2 Study II

The flowchart of patient participation is shown in Figure 11. Patient characteristics are shown in Table 5. As measured by the four selected CANTAB tests, cognitive impaired was present in 34 % at three months, 18 % at six months, and 16 % of patients at twelve months after discharge in this cohort. There were no significant, nor any clinically relevant correlation between patients' subjective score on the CFQ and their cognitive performance as measured by the four different CANTAB tests using Spearman's rank correlation (3 months r=-0.134-0.207, p>0.05, at 6 months r=-0.106-0.257, p>0.05, and at 12 months after discharge r=-0.070-0.109, p>0.05) (Figure 12). In line with **study I,** there was a significant correlation between psychological distress and patients' self-rated cognitive function (r=0.372-0.710, p=<0.001-0.023) throughout the follow-up period (Figure 13).

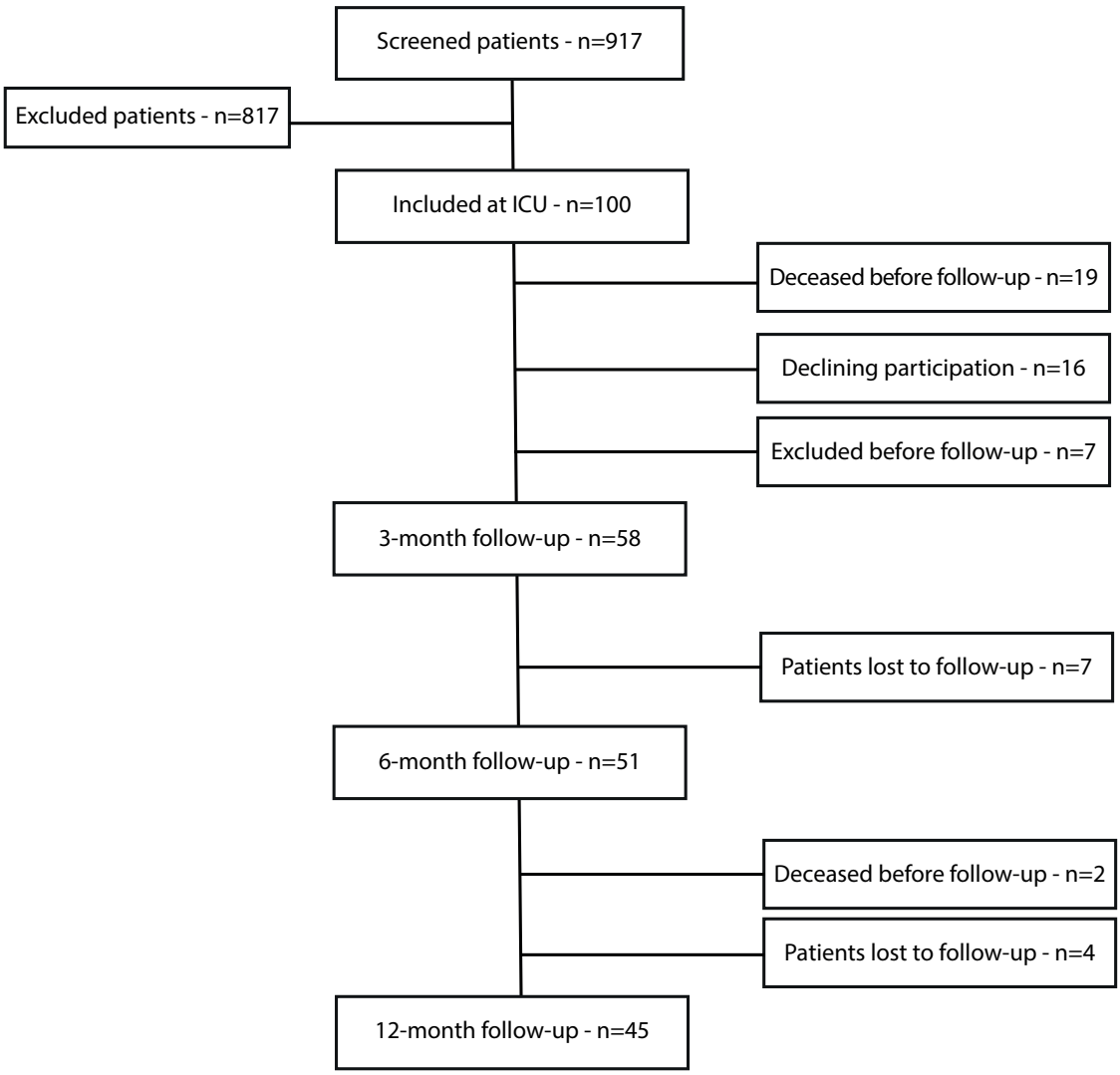

Figure 11: Flowchart of patient follow-up.

		Follow-up cohort at 3-months (N=58)
Age - yr, median (IQR)		54 (41-64)
Male sex - no. (%)		44 (76)
Level of education - no (%)	Primary	15 (26)
	Secondary	24 (41)
	Tertiary	19 (33)
Nicotine abuse - no. (%)		22 (38)
Comorbidity - no. (%)	Cardiovascular	18 (31)
	Respiratory	7 (12)
	Gastrointestinal	3 (5)
	Diabetes	6 (10)
	Cancer	8 (14)
	Immunological	2 (3)
	Neurological	4 (7)
APACHE II score - median (IQR)		26 (22-30)
SAPS III score - median (IQR)		48 (40-53)
Sepsis/septic chock (Sepsis 3) - no. (%)		42 (72)
Mechanical ventilation - no. (%)		44 (76)
Delirium - no. (%)		17 (29)
Duration of ICU stay - days, median (IQR)		4.45 (2-8.5)

Table 5: Patient characteristics. *IQR, interquartile range; APACHE, Acute Physiology and Chronic Health Evaluation; SAPS, Simplified Acute Physiology Score; Level of education, according to the Swedish national school system (Primary – ages 6-15, Secondary – ages 15-18, Tertiary – University level).*

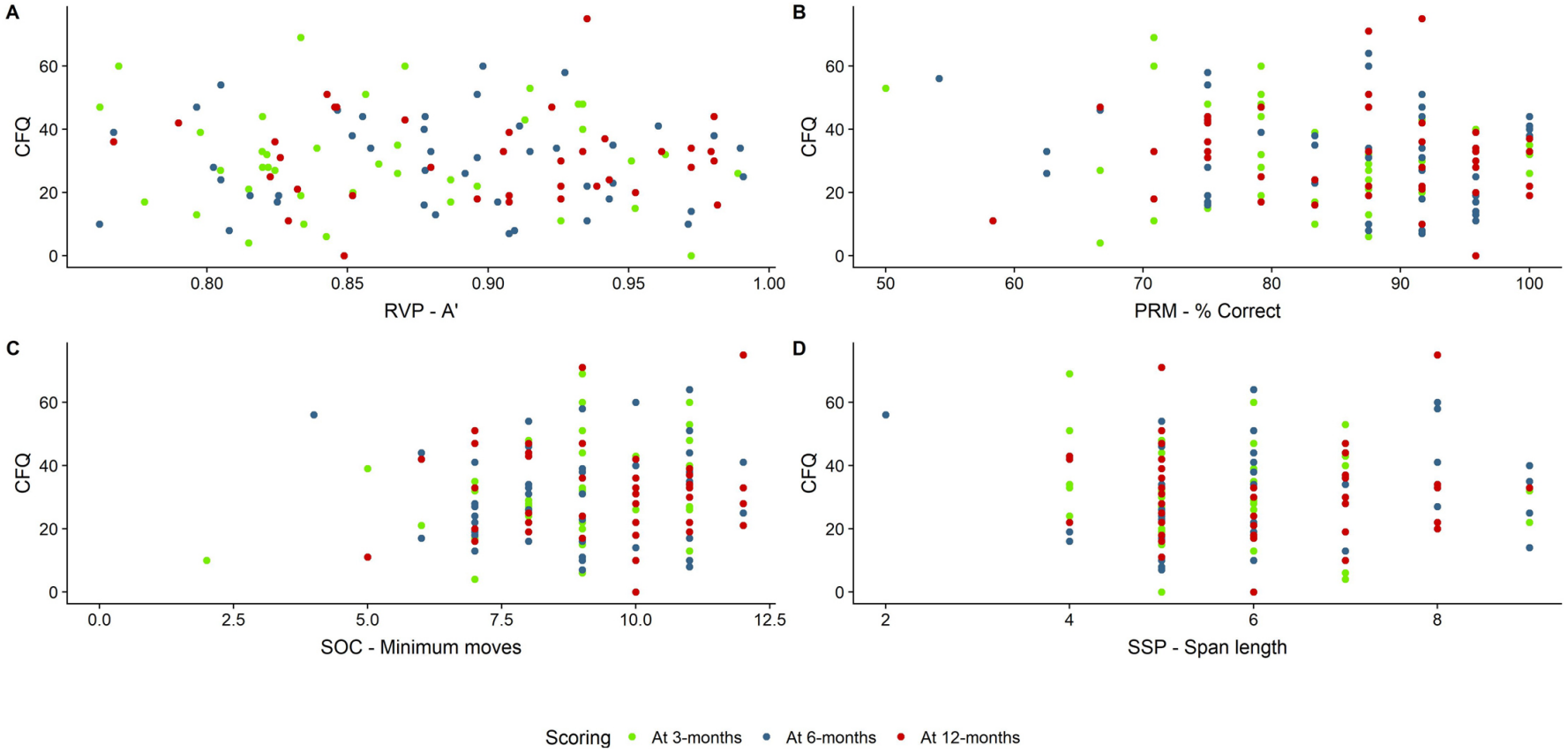

Figure 12: Correlation between subjective and objective cognitive function. *Individual patients score are plotted A) CFQ scores and % correct on the PRM test B) CFQ scores and the outcome measure RVP A' C) CFQ scores and number of tests with minimum moves completed in the SOC test D) CFQ scores and span length achieved on the SSP test*

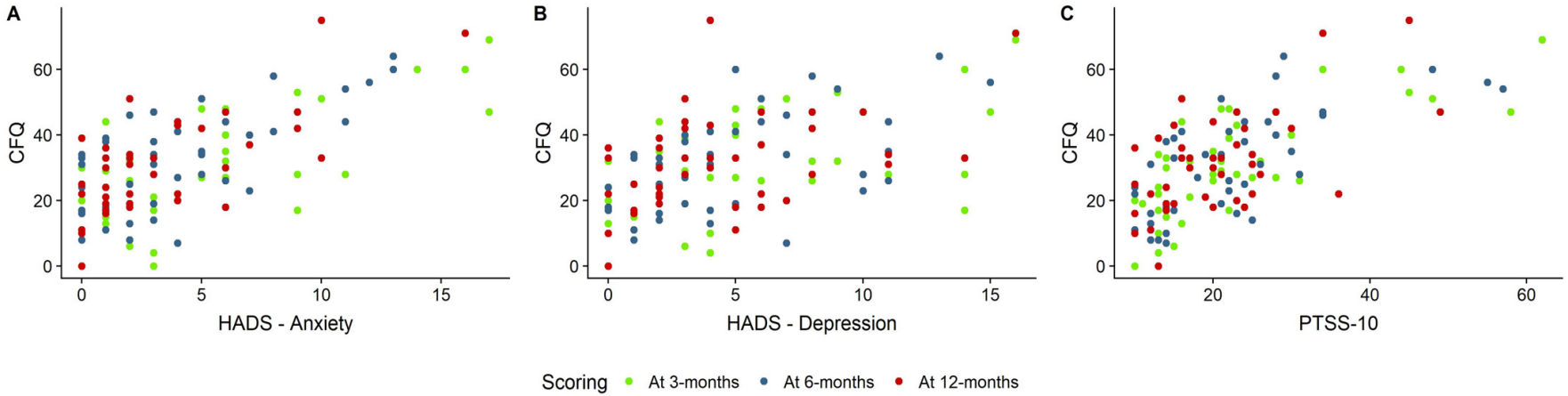

Figure 13: Correlation between subjective cognitive function and psychological distress. *Individual patients score are plotted A) CFQ scores and HADS anxiety subscale score B) CFQ scores and HADS depression subscale score C) CFQ scores and PTSS-10 score. CFQ, The Cognitive Failures Questionnaire; CANTAB, Cambridge Neuropsychological Test Automated Battery; PRM, Pattern Recognition Memory; RVP, Rapid Visual Information Processing; SOC, Stockings of Cambridge; SSP, Spatial Span; HADS, Hospital Anxiety and Depression Scale; PTSS-10, Post-traumatic Symptom Scale.*

5.3 Study III

Flowchart and patient characteristics are shown in Figure 11 and Table 5. Plasma levels of HMGB1 were significantly elevated in ICU survivors, compared to a healthy reference group (Figure 14a). Other investigated proinflammatory cytokines, including TNF-α, IL-1β, IL-6, IL-8, IL-12p70, and IL-10, were not elevated at three months after ICU discharge (N=18, data not shown). The evolution of HMGB1 levels differed between patients as shown in Figure 14b, and plasma HMGB1 levels increased in the majority of patients between the three- and six-month follow-up visits.

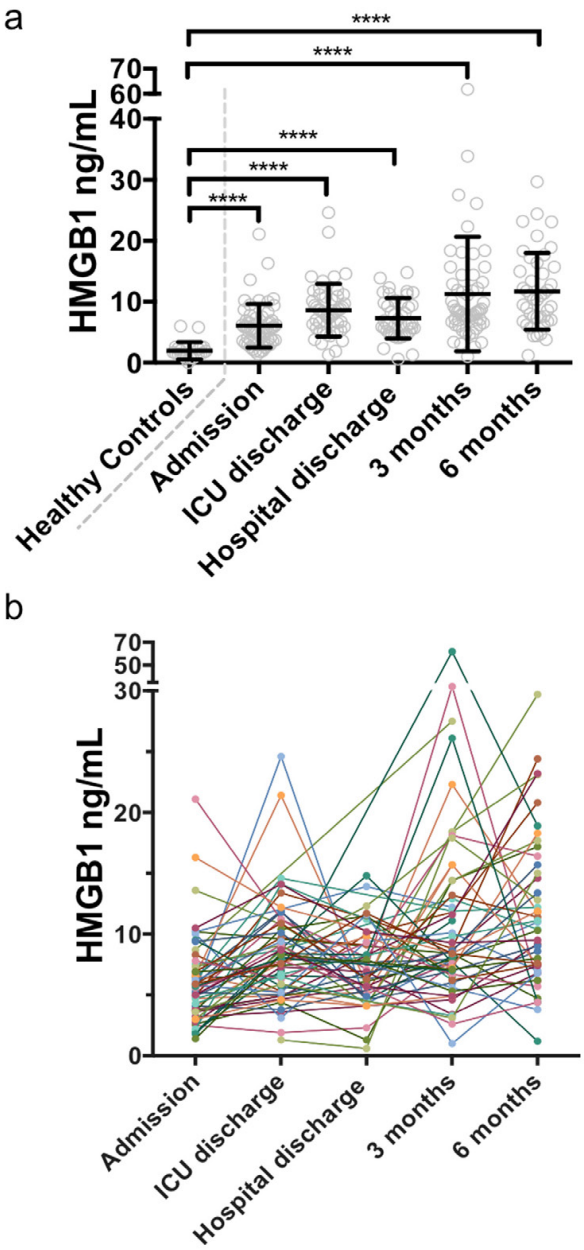

Figure 14: a) Plasma HMGB1 levels over the follow-up period. *In healthy controls 2.0±1.4 ng/mL (N=22). In ICU survivors, 6.1±3.6 ng/mL (N=56), at ICU-discharge 8.6±4.3 ng/mL (N=50), at three months after ICU discharge 11.3±9.4 ng/mL (N=56), and at six months 11.7±6.3 ng/mL (N=43). Numbers indicate mean plasma HMGB1 values ± standard deviation. Individual values are plotted.*

****- p<0.0001.

b) Patients individual evolution of plasma HMGB1 levels. *Different colors and lines represent individual patients.*

Patients' z-score derived from gender and age-matched British normative data provided by the CANTAB manufacturer, show a significant reduction in their performance on the RVP test that measure sustained attention at both three ($p<0.0001$) and six months ($p=0.04$) (Figure 15 a, b). RVPA' test score also significantly improved between three and six months ($p=0.01$), whereas there is no significant change regarding the other tests (SSP, SOC, PRM).

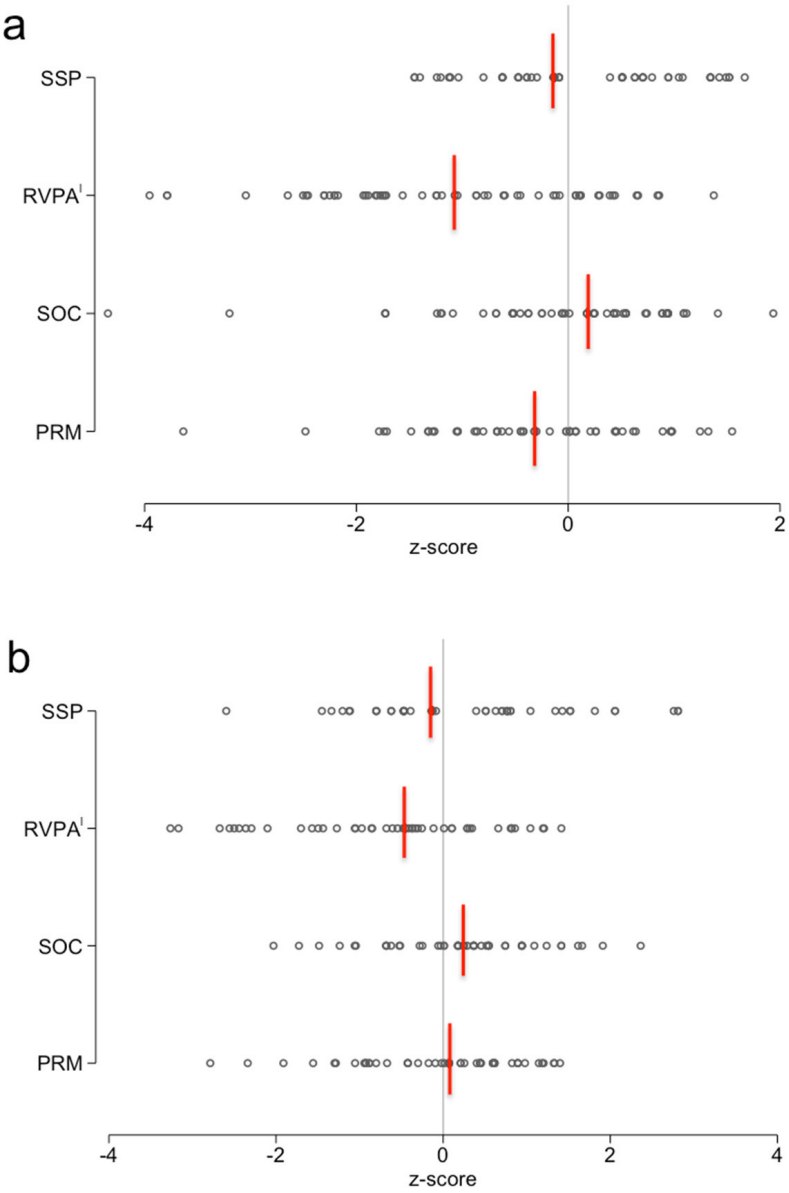

Figure 15: Patients individual z-score at a) three months and b) six months after ICU discharge. *The median z-score of the tests is indicated by red lines. Patients´ individual z-score is plotted. PRM, pattern recognition memory (% correct); RVP, rapid visual information processing (RVPA'); SOC, Stockings of Cambridge (min moves); SSP, spatial span (span length).*

Plasma levels of HMGB1 at three and six months were significantly associated with RVPA' scores, i.e. an increase in plasma HMGB1 was associated with a decrease in RVPA' score (Figure 16) both crude (p=0.03) and after adjusting for age, gender and education level (p=0.02). There was no significant association between plasma HMGB1 levels and the test scores on the other cognitive tests (SOC, PRM, SSP).

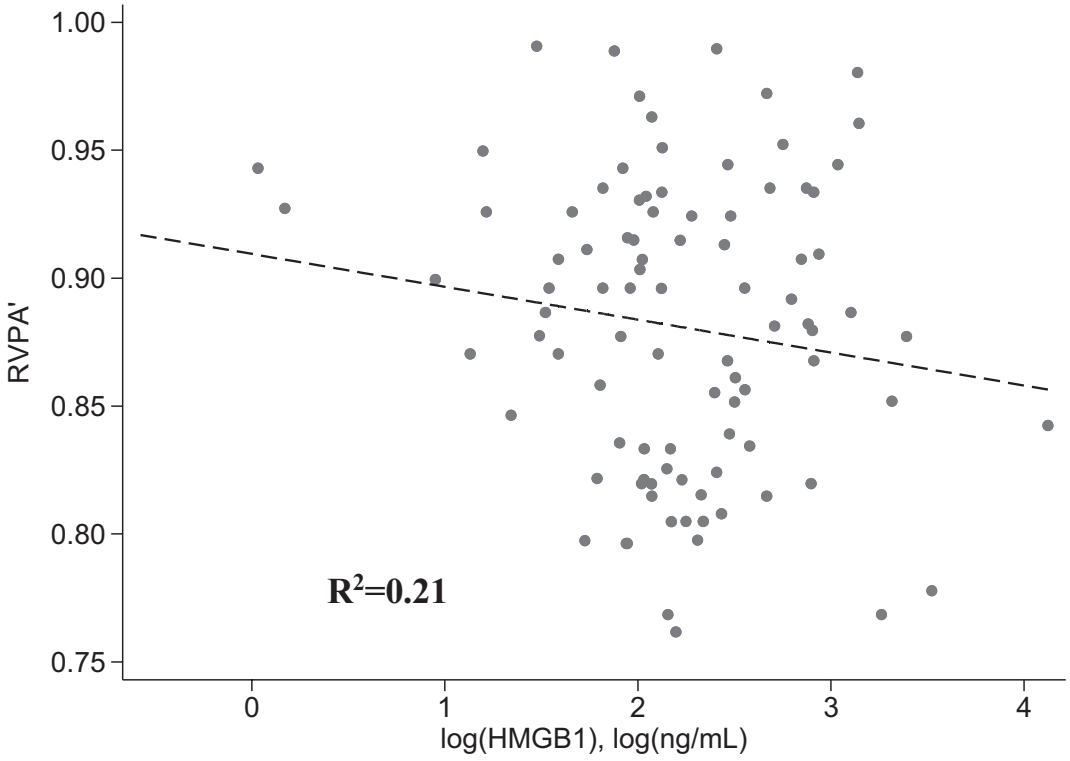

Figure 16: HMGB1 and sustained attention in linear mixed models adjusted for age, gender, and education level. *Log transformed HMGB1 levels at three and six months on the x-axis and RVPA' score at three and six months on the y-axis. Individual patient values are plotted. R^2=0.21, p=0.02. RVP, rapid visual information processing*

5.4 Study IV

Flowchart and patient characteristics are found in Figure 11 and Table 5. Patients' physical performance significantly improved over the follow-up period (p<0.01-0.02). There was no significant association between plasma HMGB1 levels and physical performance at follow-up (Table 6). Patients performed worst on TST and HST compared to the reference values (Table 7), and 16 % of patients under-performed on all tests at three months, and 12 % at six months.

Physical tests	Coef.	95 % CI	p-value
Handgrip strength right			
HMGB1 (3+6 months)	- 0.46	-2.36-1.43	0.63
Time (6 months)[a]	1.79	0.25-3.34	0.02
Handgrip strength left			
HMGB1 (3+6 months)	0.58	-1.43-2.59	0.57
Time (6 months)[a]	1.29	-0.42-3.00	0.14
Timed stands test			
HMGB1 (3+6 months)	-0.39	-3.33-2.21	0.69
Time (6 months)[a]	-3.84	-6.39-1.28	<0.01
6-min walk test			
HMGB1 (3+6 months)	12.4	-23.9-48.6	0.50
Time (6 months)[a]	42.0	11.4-72.5	<0.01

Table 6: Plasma HMGB1 levels and physical test scores. *Plasma HMGB1 levels and physical performance at three and six months were analyzed in a linear mixed model adjusted for age and gender. Time (6 months) shows the effect of time on the specific test.* [a] *= reference 3 months*

	Number of patients with reduced physical performance score	
	Follow-up at 3-months	**Follow-up at 6-months**
6-min walk test - no. (%)	17 (31)	11(25)
Handgrip-strength test right - no. (%)	20 (35)	16 (32)
Handgrip-strength test left - no. (%)	17 (30)	16 (32)
Timed-stands test - no. (%)	32 (55)	23 (46)
Under performed at all tests – no. (%)	9 (16)	6 (12)

Table 7: Number of patients with reduced physical performance at the three and six month follow-up. *Reduced physical performance was defined as scoring below the 95% confidence interval in age and sex adjusted reference values.*

6 DISCUSSION

6.1 Methodological considerations

6.1.1 Validity

The applicability of epidemiological studies depends on its internal and external validity. Internal validity is defined as to what degree observations are correct and not due to methodological errors or confounding factors, i.e. that the results found in a study represent the truth. External validity represents the generalizability to which the result found in a study can be applied to other study populations. If a study lacks internal validity, external validity becomes irrelevant.

The validity is influenced by sources of errors frequently found in epidemiological studies and consists of systematic and random errors. Systematic errors include selection bias, misclassification, and confounding.

Selection bias occurs when the selection of the study population creates the impression that two variables are related even though they are not associated in the target population. In cohort studies where data is collected prospectively, inclusion and exclusion is not based on future outcomes, which reduces the risk of selection bias. However, in follow-up studies, there is a risk of the unhealthy survivors (i.e. those with most problems die or are unable to participate in follow-up) and healthy survivors (i.e. those with no problems resume to normal life and work, and therefore do not attend follow-up or send back questionnaires) bias. This selection bias could be present in **study I-IV** as all are follow-up studies.

Misclassification or information bias occurs when exposure or outcome are inaccurately recorded. These misclassifications can appear equally (non-differential) or not equally (differential) between groups in a study. The definition of sepsis and diagnose of delirium in **study I and III** are both liable to misclassification.

Confounding is one of the major threats to internal validity in epidemiological studies. This occurs when a factor that is independently associated with both the exposure and outcome affect the association. Confounders can be handled through randomization, restriction, and matching. It is also possible to adjust in statistical analysis for potential known confounders through stratification or multivariate analysis. In **study I-IV,** strict exclusion criteria were set to limit the influence of potential confounders. Adjustments for confounders in the statistical analysis were also made in **study I-IV**. We cannot, however, as with any observational study, rule out the risk of bias due to unmeasured confounders.

Even when a study is perfectly designed and systematic errors are minimized, there will always be a risk of chance findings. These results are always unknown, unpredictable, and referred to as random errors. In **study I-IV,** we set a significance level of $p<0.05$, with a 5 % risk of type I errors, i.e. finding an association or difference where there is none. The relatively small sample sizes of the studies conducted here also give an inherent risk of type II errors, i.e. not finding an association or difference when there is one.

In summary, given the strict study design of the studies conducted here, a higher internal validity may be assumed. The external validity or generalizability should, however, be interpreted with caution, considering that it is single-center studies, with relatively small sample sizes, from a general ICU with a high proportion of trauma patients, which results in a relatively young study population and a higher proportion of men.

6.1.2 Self-assessment

Self-evaluation is difficult, and there are several pitfalls that people tend to succumb to when they assess their abilities. One may presume that self-assessment should be unbiased and closely connected to our genuine abilities as we are continuously given real-life information on how we perform various tasks. Nevertheless, many studies have found our ability to self-assess to be biased, especially when it comes to self-estimating functions such as cognitive ability (44). The main distortion in self-assessment is the *better-than-average effect*, which is defined as the tendency of individuals to assess various abilities as being above average (140). Another well-known bias is the Dunning-Kruger effect that focuses on differences in metacognition (i.e. being aware of one's awareness). It includes the fact that people who generally overperform, underestimate their ability and those who underperform, overestimate theirs (141). Furthermore, regarding CFQ that was used in **study I and II,** there is also an age-CFQ paradox, where older people that have more cognitive impairments rate themselves with low (i.e. less cognitive difficulties) score. In order to answer the CFQ, one needs to remember what one has forgotten, something that is difficult in those cognitively worse off (142). This is an inherent methodological problem with this type of self-rated cognitive ability assessment tools, leading to misidentification of cognitive impairments by self-rated screening tools. In addition, there is also a "complaint hypothesis" when assessing both cognitive and psychological problems (143). If a patient feels a need for help, no matter the reason, they increase their symptoms when self-evaluating, something that is common in clinical follow-up, where the score on the different tests determines the need for intervention. In both cognitive and mental impairments,

a discrepancy between the individuals' self-perception and the actual deficits the clinician intends to assess may result in misjudge of the need for rehabilitation, or the risk of early termination of rehabilitation although needed, in both cases with negative consequences for patients' recovery. Structured, unbiased screening tools are, therefore, of great importance in ICU follow-up.

6.1.3 HMGB1

To increase the reproducibility and allow for comparison, the use of reliable methods for detecting HMGB1 is essential. We measured HMGB1 with the current gold standard ELISA from Shino-test Corporations (144). Several studies have instead used western blot, a method that has its limitations in the quantification of HMGB1 concentrations. As mentioned in the introduction, HMGB1 can complex-bind with several other pro-inflammatory cytokines. The ELISA form Shino-test Corporations uses acidic buffers, to promote dissociation to bound molecules from HMGB1. In western blot targeting HMGB1, a filter column to discard larger molecules early in the assay is commonly used, but since HMGB1 usually is complex bound *in vivo*, there may be a risk of miscalculation and false low readings. None of these assays distinguish between hyperacetylated HMGB1 or its redox forms. So far, only mass spectrometry allows such analysis. The mass spectrometry technique has proven difficult, and in the last year suffered from scientific misconduct explicitly related to the measurements of HMGB1 reported from a British lab. The identification of different biologically active forms of HMGB1 is, therefore, a challenge that lies ahead.

The future clinical use of HMGB1 measurements would be greatly facilitated by the development of methods for bedside testing. The present ELISA is time-consuming, however, at the 9th international DAMP and alarmin conference in Japan, which was held in November 2019, a new latex-immunoassay from Shino-test Corporations was presented, and forecasted to soon be available for purchase. The latex-immunoassay, which performs HMGB1 concentration measurements in 10 minutes on regular hospital instruments, promises to reform the future of clinical HMGB1 testing.

6.2 Interpretation of findings

Intensive care unit survivors are an increasing patient population that suffers from new onset of psychological, physical, and cognitive impairments. This purpose of this thesis was to identify possible new triggering factors in the development of PICS.

In study I, we found no association between clinically diagnosed sepsis and delirium, and patients later self-rated cognitive function, and there may be several explanations to be found here. First, it is important to recognize that both sepsis and delirium are definitions that have evolved in order to understand and categorize physiological conditions. Hence, one may acknowledge the complexity of different immune responses and that the clinical definitions may not be accurate enough considering the various conditions that occur during intensive care. One patient may have sepsis for two hours, whereas the neighbor has it for two weeks, and they both fall into the same category, and, therefore, there may be significant differences in the immune response between such individuals. The same is true for delirium, which might be a reason why only the duration of delirium has proven to be associated with later cognitive impairment (7). Secondly, we used self-rated cognitive function in study I, and as we in study II found no correlation between patients' subjective and objective cognitive function, there might still be an association between sepsis, delirium, and cognitive function, only that we did not capture it here in this setting. The strong correlation between subjective cognitive function and psychological distress was consistent in the two patient cohorts. What comes first is difficult to tell. Do patients get depressed because they are experiencing cognitive failures, or is depression affecting cognition? For the results from Study I and II, it also appears that psychological distress is only weakly correlated to worse objective cognitive function, but strongly correlated to worse subjective cognitive function. This implies that subjective cognitive performance also is a relevant patient outcome when it comes to rehabilitation and the ability to work, or, maybe, that it merely mirrors the psychological distress experienced.

As mentioned in the introduction, relevant measurements of cognitive function are a challenge for the ICU community. Neuropsychological test batteries are time-consuming and require the presence of a test conductor. The CFQ has, therefore, gained interest as a simple tool for cognitive screening, as it can be filled in at home or easily at follow-up. Notably, although it has been used as a measure of cognitive function in ICU follow-ups (43, 145), we show in study II the lack of clinically relevant correlation between patients' self-perceived cognitive failures and objective cognitive function assessed with neuropsychological tests. The difficulties with self-assessments have been discussed earlier in this section. Nevertheless, we cannot say that CFQ is a bad instrument for measuring patients' cognitive failures in everyday-life, merely that it in ICU survivors do not reflect objectively measured cognitive impairments commonly found in this patient

population. Both parts of cognition may be of relevance for the patient as they reflect different aspects of patients' lives. The experience of cognitive failures in every-day life will most likely affect social life and the ability to perform at work. The same goes for cognitive impairment, and even though patients may not suffer from the cognitive impairment, family, and social network may.

The potential link between inflammation and the development of PICS continues to intrigue us. The problem with inflammation is usually not why or how it starts, more how often it fails to resolve. After the initial trigger of the immune response, affected tissues should restore to normal function, and the inflammation subside. When this fails, non-resolving inflammation appears (57).

In order to understand the causes of PICS, and especially cognitive impairment, several studies have investigated possible predictors, such as sepsis and delirium (7, 36). Results, however, have been contradictory (13). A possible mechanism in the development of cognitive impairment after intensive care shown in this thesis is the potential pro-inflammatory effect of HMGB1. We found that plasma HMGB1 levels were elevated for up to six months after ICU discharge, a novel discovery. The kinetics observed here also support earlier studies of HMGB1 being a late mediator of inflammation (74, 122, 123). Many studies of HMGB1 in this context have been in sepsis patients or experimental sepsis models. We here, however, observed no difference in late plasma HMGB1 levels between patients diagnosed with sepsis or not at the ICU. This could be, as mentioned earlier, a result of the clinical definitions not being sufficiently precise, but most likely that both sterile and infectious stimuli triggers the same response regardless of the source.

Measuring HMGB1 in the acute phase as a predictor of long-term outcome has proven difficult (146, 147). However, a recent paper by Ottestad *et al.* in trauma patients observed that not the initial plasma HMGB1 levels but a second wave of elevated plasma HMGB1 with onset a few hours after the trauma, was associated with worse outcomes (fewer ventilator-free days). As HMGB1 is released both passively (during necrosis and pyroptosis) and actively (from stressed and activated immune cells), one may guess this second wave may come from the latter.

The source of the exceptionally late, elevated levels of HMGB1 in ICU survivors found here remains an enigma. It is tempting to speculate that it comes from actively released HMGB1, as other markers of cell destruction such as lactate dehydrogenase was low at follow-up (unpublished data). Additionally, considering the relatively short half-life of HMGB1, which varies between minutes and a few hours (148, 149), this also speaks for an ongoing, active release of HMGB1. Active release of HMGB1 is dependent on the translocation of HMGB1 from the nucleus to the cytosol. As mentioned earlier, the release is mediated through posttranslational modifications, with hyperacetylation of the two NLS sites (75), which translocate HMGB1 to the cytosol. Hyperacetylation of the NLS sites of

HMGB1 is in infection a result of activation of the JAK-STAT pathway (150). It would be intriguing to know if the JAK-STAT pathway is activated in this late mediated inflammation, but as we in study III and IV did not have the possibility to measures the degree of acetylation or redox form, one may only speculate in what form the plasma HMGB1 found here is.

We found in study III that levels of HMGB1 were associated with sustained attention measured with RVP, one of the four cognitive tests chosen. Furthermore, we found that sustained attention was significantly impaired in our cohort compared to normative data, but not the other functions measured (i.e., executive function, visual memory, and working memory). This may indicate that sustained attention is the cognitive function most affected in ICU survivors tested here, or that the other three tests are limited in identifying cognitive impairment in this cohort. These findings might be an explanation of why we only observed an association between sustained attention and plasma levels of HMBG1, and not the other tests.

The lack of association between physical performance and plasma HMGB1 levels in study IV might have several explanations. The study design was predominantly constructed to identify new onset of cognitive impairment and not physical impairment. We can, therefore, not with certainty, conclude that the physical impairments measured here are of recent onset, and possibly associated with effects related to elevated levels of HMGB1. Furthermore, earlier studies on HMGB1's association with muscle dysfunction have focused on local processes in muscle biopsies (129, 130), and it may be that plasma HMGB1 levels do not accurately reflect the intracellular milieu in muscle tissue.

6.3 Limitations

As ICU admissions predominantly are due to unexpected, acute illness, it precludes the assessment of baseline data. We, therefore, could not measure baseline cognitive function or baseline physical performance in the studies. In study II-III, however, we excluded patients with a history of cognitive impairment and screened patients with the IQCODE in which the patients' next of kin score symptoms of cognitive decline, where patients with significant symptoms were excluded. We did this in order to be able to identify the new onset of cognitive impairments as a part of PICS, rather than to study preexisting cognitive impairment. As mentioned earlier are all four studies within this thesis follow-up studies, and the risk of selection bias in responders should, therefore, be considered. We also cannot rule out the fact that patients' motivation to perform could have varied in between tests, due to fatigue or other emotional factors, considering that both the cognitive and physical tests demand some focus and willpower.

7 FUTURE PERSPECTIVES

Many challenges lie ahead for the scientific ICU community for improving patients´ life after ICU. Structured, standardized test methods to identify ICU survivors at risk or in need of rehabilitation are needed. Possible future interventions to attenuate the development of cognitive impairment in this growing patient population would be of great benefit.

A clinically feasible way to measure HMGB1 with the promising new latex immune-assay would enable practical measurements that could help in our future understanding of HMGB1-biology in ICU patients. Another important step in order to understand the pathophysiology would be to identify the degree of acetylation and which redox forms that is released, a technique that regrettably is not easily available at the moment. It is tempting to speculate that further studies of lysophosphatidylcholine, the lipid that mediates the active exocytosis of HMGB1 (76), could help us understand whether the HMGB1 released months after intensive care is actively or passively released.

Four things have successfully targeted HMGB1 up to 24 h after onset of experimental sepsis and improved survival: anti-HMGB1 antibodies, vagal nerve stimulation or α7nAChR-agonists, and monoclonal antibodies against RAGE (99, 151-153). Considering that these therapeutic approaches attenuate HMGB1, and based on the experimental results of alleviation of cognitive dysfunction (71), future possible anti-HMGB1 therapy might improve cognitive outcomes in ICU survivors. This, however, remains to be studied.

8 CONCLUSION

After critical illness, many patients suffer from debilitating long-term sequels that reduce quality of life and prevent the return to pre-morbid levels of function. Tools to easily predict, diagnose, and treat these important sequels after critical care are lacking. This thesis aims to identify clinical predictors of adverse cognitive outcomes, map aspects of the underlying biology, and ideally suggest molecular targets amenable to therapeutic intervention. In conclusion, this effort aims to improve our understanding of cognitive impairment after critical illness and hopefully provide new knowledge that may contribute to improving patients´ life after ICU.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Allt fler överlever idag intensivvårdsbehandling. Frågan är - med vilken livskvalitet? Vid Karolinska Universitetssjukhuset i Solna studerar man följderna av intensivvård, för att på så sätt underlätta rehabiliteringen efter livshotande sjukdom och intensivvård. Man har då funnit att en betydande del av patienterna uppger att de även månader efter intensivvård har påverkad kognitiv funktion, såsom problem med minne och uppmärksamhet. Förutom de kognitiva svårigheterna lider även upp till hälften av psykiska och fysiska problem. Dessa symtom kallas tillsammans för post-intensive care syndrome (PICS). Förutom att påverka patienterna i deras vardagsliv så försvåras även återgången till ett normalt liv och arbete. Orsakerna till denna ökade incidens av psykiska, fysiska och kognitiva problem är ännu inte kända. Blodförgiftning och akut förvirring (delirium) under intensivvård har i vissa studier visat på ett samband med senare kognitiva problem. I musmodeller av blodförgiftning har man hittat ett samband mellan ett pro-inflammatoriskt protein, High Mobility Group Box 1 (HMGB1), och kognitiv dysfunktion. Hur och om HMGB1 direkt eller indirekt påverkar kognitiv funktionsnedsättning är oklart, men det finns teorier att HMGB1 i kombination med andra pro-inflammatoriska proteiner aktiverar immunceller i hjärnan (mikroglia), vilket kan leda till neurotoxiska effekter. Intressant nog har det även visats sig att HMGB1 är förhöjt i blodet under en mycket längre tid än andra pro-inflammatoriska proteiner efter intensivvård. I musmodellen har man dessutom kunnat hämma HMGB1 flera dagar efter episoden av blodförgiftning, med förbättrad kognitiv funktion som resultat. Det här är något som öppnar upp för möjliga, framtida terapier.

Denna avhandlings inledande arbete undersökte sambandet mellan blodförgiftning och delirium under vårdtiden på intensiven och senare självskattad kognitiv funktion, samt sambandet mellan psykiska problem och självskattad kognitiv funktion. Vi fann där ett samband i det senare fallet men inget samband med blodförgiftning och delirium. En orsak till detta skulle kunna vara att patienter har svårt att skatta den egna faktiska förmågan, framförallt den kognitiva. Den påföljande studien undersökte därför om patienters självskattade kognitiva funktion överensstämmer med objektiv kognitiv funktion testad genom formella neuropsykologiska tester. Vi kunde då visa att så inte var fallet, och man ska vara försiktig när man tolkar resultat från subjektivt skattade svarsformulär från intensivvårdspatienter. För att vidare kunna förstå orsaken till de svårigheter efter intensivvård som patienter lider av undersökte vi sambandet mellan den tidigare beskriva HMGB1 samt kognitiv funktion, tre och sex månader efter intensivvård. Vi fann då att nivåer av HMGB1 i blodet var signifikant associerat med kognitiv funktionsnedsättning i form av patienters sänkta förmåga till uppmärksamhet. Detta är i linje med de resultat som kommit från experimentella musmodeller.

Sammanfattningsvis syftar denna avhandling till att förbättra vår förståelse för kognitiv dysfunktion efter kritisk sjukdom och förhoppningsvis ge ny kunskap för att förbättra diagnos och behandling i denna stora patientpopulation.

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