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Heparin-binding protein and organ failure in critical illness.

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"Excellent intensive care requires meticulous attention to detail without losing perspective"

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Abstract

Background For patients severely ill enough to require care in an intensive care unit (ICU), both the disease itself (e.g. bacteria in the blood in sepsis or fractures after trauma) and effects of the immune system can cause circulatory, pulmonary, or renal dysfunction. Leukocytes play a dominant role in the immune system. When activated they release a range of small proteins with different properties Heparin-binding protein (HBP) being one of these proteins, has many functions, including to increase vascular permeability. Heparin-binding protein causes plasma leakage from blood vessels into surrounding tissue (oedema), which can lead to organ dysfunction depending on the site and degree of oedema formation. Increased concentration of HBP in plasma is associated with failing circulation and lung function in subgroups of critically ill patients.

Aims We investigated the possibility of using concentration of HBP in plasma for predicting circulatory, respiratory or renal failure in an ICU population with mixed diagnosis. We assessed concentration of HBP in alveoli in ventilator induced lung injury (VILI), and finally assessed elimination of HBP in urine and effluent fluid from continuous dialysis.

Methods In Papers I and II, HBP concentration in plasma was measured in 278 patients on admission to ICU. Sequential organ failure assessment (SOFA) scores and acute kidney injury (AKI) stage were recorded daily. In Paper III HBP concentration in bronco-alveolar fluid was measured in a pig model of ventilatory induced lung injury, in 16 healthy volunteers and in 10 intubated ICU patients. In Paper IV plasma and urine concentration of HBP was measured in 8 healthy volunteers and 20 burn ICU patients. In addition, HBP was sampled in plasma and effluent fluid in 32 ICU patients on continuous renal replacement therapy (CRRT).

Results In Paper I, patients developing circulatory failure (circulatory sub-score of SOFA = 4) had higher plasma concentration of HBP compared to those who did not (median(IQR)ng/ml) (63.5(32–105) vs 36.4(24–59)) p<0.01), and patients developing respiratory failure (P:F ratio < 27) had higher HBP concentration than those who did not (44.4(30-109) vs 35.2(23-57) p<0.01). Discriminatory capacity was (ROC AUC (95%CI)) (0.65 (0.54–0.76)) for circulatory failure and (0.61(0.54–0.69)) for respiratory failure. In Paper II, patients developing renal failure (AKI stage 2-3) had higher plasma concentration of HBP compared to those who did not (72.1 (13.0–131.2) vs 34.5 (19.7–49.3) p<0.01). Discriminatory capacity for AKI stage 3 was 0.68(0.54-0.83) (ROC AUC (95%CI)). In the subgroup with severe sepsis, it was 0.93 (0.85–1.00). In Paper III, HBP concentration in bronchoalveolar lavage was higher in pigs subjected to injurious ventilation over 6 hours ventilation compared to controls (1144(359–1636) vs 89(33–191) p=0.02) (median(IQR)ng/ml). The median HBP concentration in bronchoalveolar lavage from healthy volunteers was 0.90(0.79– 1.01) compared to 1959(612–3306) from intubated ICU patients (p < 0.01). In Paper IV, renal clearance of HBP was 0.19 (0.08-0.33) in healthy individuals and 0.30 (0.01-1.04) (median, IQR, ml/min) in burn ICU patients. Clearance of HBP was higher in burn patients

with increased cystatin C (0.45(0.15-2.81) vs. 0.28(0.14-0.55) p=0.04). Starting CRRT did not alter plasma concentration of HBP (p=0.14). Median HBP concentration in effluent fluid on CRRT was 9.1 ng/ml (7.8-14.4).

Conclusions Papers I and II*:* There is an association between high concentration of HBP in plasma on ICU admission and circulatory, respiratory and renal failure. For the individual patient, the predictive value of a high HBP concentration is low, with the possible exception of renal failure in septic patients. Paper III*:* HBP concentration in alveoli increases in pigs subjected to injurious ventilation. HBP concentration in alveoli of intubated ICU patients ventilated protectively is elevated to similar levels, a factor of approximately 1000 times higher than the concentration seen in healthy controls. Paper IV*:* In healthy study participants, renal clearance of HBP is low. In critically ill burn patients with impaired renal function, clearance of HBP is increased. Starting CRRT in critically ill patients does not alter plasma concentration of HBP. Still, HBP is found in the CRRT effluent fluid, and concentration does not appear to be dependent on plasma concentration.

Keywords Heparin-binding protein, Critical care, Shock, Acute respiratory distress syndrome, Acute kidney injury, Ventilator induced lung injury, Renal clearance

Original papers

Paper I. Tyden J, Herwald H, Sjoberg F, Johansson J.
Increased Plasma Levels of Heparin-Binding Protein on Admission to Intensive Care Are Associated with Respiratory and Circulatory Failure. *PLoS One 2016; 11: e0152035.*

Paper II. Tyden J, Herwald H, Hultin M, Wallden J, Johansson J.
Heparin-binding protein as a biomarker of acute kidney injury in critical illness. *Acta Anaesthesiol Scand 2017; 61: 797-803.*

Paper III. Tyden J, Larsson N, Lehtipalo S, Herwald H, Hultin M, Wallden J, Behndig AF, Johansson J. Heparin-binding protein in ventilator-induced lung injury. *Intensive Care Med Exp 2018; 6: 33.*

Paper IV. Samuelsson L, Tyden J, Herwald H, Hultin M, Wallden J, Steinvall I, Sjöberg F, Johansson J. Renal clearance of heparin-binding protein and elimination during renal replacement therapy: Studies in ICU patients and healthy volunteers. *(Accepted for publication)*

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Abbreviations

AKI	Acute kidney injury
ALI	Acute lung injury
ANG	Angiopoetin
ARDS	Acute respiratory distress syndrome
AUC	Area under the curve
BALF	Bronco-alveolar lavage fluid
BNP	Brain natriuretic peptide
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CRRT	Continuous renal replacement therapy
CVVHDF	Continuous veno-venous hemodiafiltration
DAMP	Danger associated molecular pattern
DNA	Deoxyribonucleic acid
ED	Emergency department
ELISA	Enzyme-linked immunosorbent assay
GFR	Glomerular filtration rate
HBP	Heparin-binding protein
ICU	Intensive care unit
IL	Interleukin
IGFBP	Insulin-like growth factor binding protein
IQR	Interquartile range
KDIGO	Kidney disease: improving global outcome
KIM	Kidney injury molecule
LPS	Lipopolysaccharide
MDRD	Modification of diet in renal disease
NGAL	Neutrophil gelatinase associated lipocalin
PAMP	Pathogen associated molecular pattern
PEEP	Positive end expiratory pressure

P:F ratio	Arterial partial pressure of oxygen divided by fraction inhaled oxygen
RAGE	Receptor for advanced glycation endproducts
ROC	Receiver operating characteristic
SAPS	Simplified acute physiology score
SIRS	Systemic inflammatory response syndrome
SOFA	Sequential organ failure score
SP-D	Surfactant protein-D
TBSA	Total body surface area
TIMP	Tissue inhibitor of metalloproteinases
TNF	Tumour necrosis factor
VILI	Ventilator induced lung injury
WBC	White blood cell count

Introduction / Background

When illness is complicated by organ failure, the need for increased monitoring or support of vital functions may necessitate care in an intensive care unit. Failing circulation, respiration or kidney function is often caused by an "over reaction" of the body's own immune response rather than by the primary illness itself[1,2]. A new and simple way to better monitor the immune response might make it possible to earlier predict the development and severity of organ failure. Achieving this would make it possible to identify patients in need of early increased attention, and, also identify those without such a need which would help in prioritizing limited resources.

The majority of treatments provided in intensive care units are supportive and not aimed specifically at the cause of the vital organ dysfunction. Much focus is aimed at providing support while not causing further harm with this support. The close relationship between immune response and organ failure has led to many treatment attempts aimed at modulating this response. While many were promising in animal models, they have all failed to improve patient outcome, and some have even been harmful when assessed rigorously in clinical studies[3]. It seems plausible that our understanding of the immune system is too limited to adequately know how to intervene in the progression of uncomplicated illness to illness complicated by failure of one or several organs.

In this thesis, I have studied aspects of one part of the immune system: heparin-binding protein (HBP) secreted from leukocytes. I examine HBP's properties as a biomarker predicting organ failure in critically ill patients, its role in pulmonary inflammation caused by harmful use of mechanical ventilation and its elimination via urine.

The innate immune system in critical illness

The immune system can be divided into the adaptive and the innate immune system. Where the innate immune system can respond rapidly to threats and pathogens not previously encountered, the adaptive immune system is more specific but slower, and depends on activation by the innate immune system.

Many important effects of the immune system are mediated by leukocytes. The first response is triggered by contact between leukocytes and either factors related to tissue injury (referred to as damage associated molecule patterns or DAMP) or factors related to invading pathogens (referred to as pathogen associated molecular patterns or PAMP). Examples of these are extracellular DNA and lipopolysaccharide (LPS) respectively.

From birth leukocytes have surface receptors for DAMPs and PAMPs. A large number of such receptors are recognized (including toll like receptors, nucleotide oligomerization domain receptors and C-type lectine receptors) which in turn are subdivided into sub classes. Activation of these receptors triggers a large variety of responses depending on the receptor activated and the type of leukocyte activated. These include release of cytokines, triggering

of phagocytosis, antigen presentation and much more aimed at killing pathogens, clearing of dead tissue, and eventually starting repair of tissue[4].

Common causes of critical illness, such as severe infection, cardiac arrest, trauma or major surgery, are all associated with an initial massive activation of the innate immune system leading to a rise in levels of cytokines[1,4] and often a rise in the concentration of leukocytes in the blood[5]. It is common that the need for critical care support is mainly related to effects of the immune response[2] such as generalized vasoplegia or increased vascular permeability caused in part by proinflammatory mediators like TNFα and IL-1β[4].

Heparin-binding protein

Heparin-binding protein (HBP), also known as CAP37 or azurocidin, is a 27 kDa protein of the serine protease family. It is found in neutrophil granulocytes. It is prefabricated and stored in secretory vesicles and azurophilic granule[6]. Release of HBP is triggered by activation of the neutrophil by crosslinking of beta-2-integrins on the its surface[7].

When neutrophils are activated, they become adherent to blood vessel walls. Crosslinking of beta-integrins is typically achieved by contact with adhesion molecules on vascular endothelial cells, but crosslinking can also occur in the circulation by contact with PAMPs from streptococci[8,9].

HBP has many effects. It is antimicrobial[10], chemoattractant[11] and has the ability to increase vascular permeability[7].

HBP binds to proteoglycans on the endothelial surface and the resulting increase of vascular permeability is dependent on protein-kinase C and Rho-kinase pathway activation[7,12-15] (Fig.1). When binding to proteoglycans, HBP displaces kininogen that can then be converted to bradykinin, which in turn induces increased vascular permeability via the kallikrein-kinin system[16]. Presumably, the increase in permeability facilitates neutrophil migration across the vessel wall and into the tissue.

Where the release of HBP from secretory vesicles is rapid and may contribute to increasing vascular permeability within minutes of a stimuli, release of HBP from azurophilic granule is delayed and occurs when the neutrophil has migrated into the tissue.

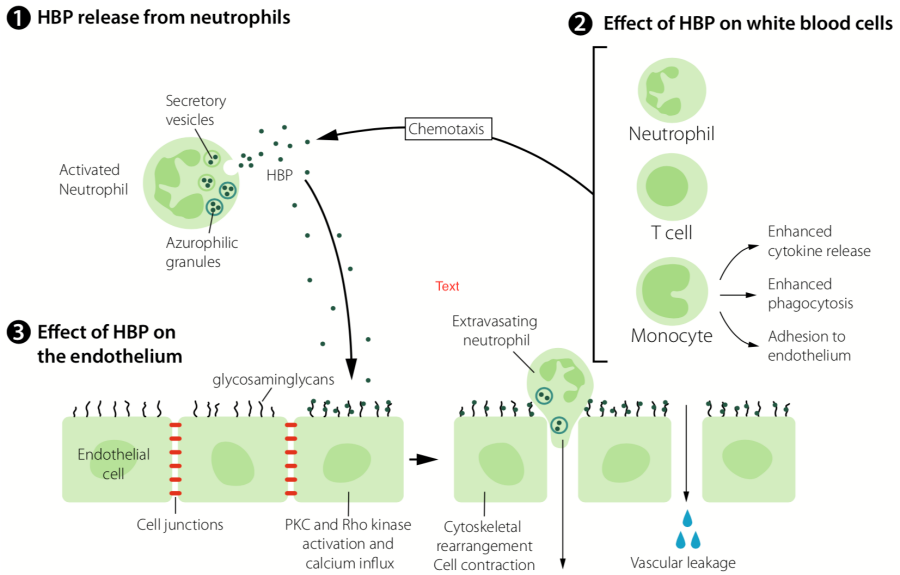

Figure 1. Schematic overview of effects of heparin-binding protein. Reprint from Fisher J, Linder A. Heparin-binding protein: a key player in the pathophysiology of organ dysfunction in sepsis. J Intern Med 2017; 281: 562-74. With permission from John Wiley and sons

Biomarkers

A biomarker is a biologic measurement that predicts a disease state, severity, risk or effect of treatment. While some biomarkers have been used in clinical practice for a long time (for example, c-reactive protein for bacterial infections and troponin for myocardial injury), there has been a recent addition of many biomarkers for clinical use especially in oncology. There is ongoing discussion about the role of biomarkers for clinical group identification or even as endpoints in clinical trials[17,18].

Biomarker use to identify organ dysfunction in critically ill patients is an growing field of research. For circulatory dysfunction, examples of biomarkers in clinical practise are lactate for anaerobic metabolism or liver dysfunction, brain natriuretic peptide (BNP) for cardiac failure and troponins for myocardial injury. The two latter are frequently used in non-critically ill patients, though their optimal application in critical illness is not yet clear[19,20].

Large efforts have been made to find robust biomarkers for sepsis. By definition, sepsis is life-threatening vital organ dysfunction, and septic shock is this with circulatory failure[21]. C-reactive protein and procalcitonin are laboratory measurements currently used as biomarkers of bacterial infections, although both have limitations in the critical care setting[22]. Biomarkers reflecting hyperinflammation, immune suppression, complement activation, hyper coagulation and neutrophil activation are currently being vigorously studied[23]. When elevated in patients with sepsis,

several of these biomarkers have been associated with disease severity and mortality, though not yet discriminating between sepsis and other causes of organ failure, and so not yet incorporated in routine clinical practice. An inherent problem with many biomarkers of disease in ICU cohorts is that while they may show promise when used to predict severe problems in an otherwise reasonably healthy cohort of patients, they are not as good for this in an intensive care unit where all patients, more or less, already have organ failure of some kind.

For acute respiratory distress syndrome (ARDS), a number of potential biomarkers have been studied. Among the more promising are receptor for advanced glycation end-products (RAGE), expressed on lung epithelium[24], angiopoetin-2 (Ang-2), an endothelial growth factor[25], surfactant protein D (SP-D), a glycoprotein reducing surface tension in alveoli[26] and interleukin-8 (IL-8), a proinflammatory cytokine[27]. Since no single biomarker (of these) has shown high diagnostic accuracy, panels including several or all of them have been tried[28] but are not in clinical use.

For acute kidney injury, the most studied biomarker is neutrophil gelatinase-associated lipocalin (NGAL)[29] released by renal tissue under stress, but also by neutrophils. While initially promising, NGAL's diagnostic usefulness in critically ill patients is now questioned[29]. Another marker of renal tissue damage, kidney injury molecule (KIM-1) has been suggested, but needs further validation[30]. Two proteins involved in regulating cell cycle arrest are showing promise as markers of acute kidney injury in patients with sepsis, tissue inhibitor of metalloproteinases-2 (TIMP-2) and insulin-like growth factor binding protein-7 (IGFBP-7)[31]. However, to date, the only biomarkers of kidney injury in routine clinical practice are creatinine and cystatin-C.

After discovering a possible new biomarker, a series of steps have been suggested to demonstrate its clinical interest[32,33]:

- Showing that the biomarker is significantly modified in the group with the outcome or disease of interest compared to relevant controls.
- Assessment of the diagnostic accuracy of the biomarker.
- Comparison of the diagnostic accuracy with existing biomarkers.
- Showing that its diagnostic accuracy improves the ability to make a clinical decision that can help patients.
- Showing that implementation of the biomarker measurement is associated with a desirable outcome.

Through all of these steps, it is also important to understand the pathophysiologic mechanisms, kinetic properties, and physiologic effects of the biomarker[32,34]. Where Papers I and II of this thesis focus on HBP in the context of the first tree of these steps, Paper III deals with one aspect of pathophysiology and Paper IV with kinetics.

Inflammation and circulatory failure

Among many components needed to maintain adequate circulation, three central ones have all been shown to be affected by inflammatory mediators, namely: venous return to the heart, cardiac contractility and perfusion pressure[35-37].

The surface of blood vessels is composed of a monolayer of endothelial cells connected by tight junctions. Stimulation of endothelial cells by endogenous or an exogenous material can, via a series of signal pathways, widen the intercellular gap by regulating the contraction of the endothelial cytoskeleton or by altering the intercellular junctions. This results in increased vascular permeability[35]. An increase in vascular permeability can, if the loss of plasma volume is great enough, cause a decrease in venous return to the heart. Several inflammatory mediators have been shown to increase vascular permeability. For example, IL-6 has been found to do so by trans signaling[38], and TNF-α via regulation of metalloproteinase-9[39].

Contraction of cardiac muscle is achieved by intracellular calcium release from the sarcoplasmic reticulum and binding to myofilaments. It is regulated by the extrinsic factors pre- and afterload, but also by intrinsic factors affecting intracellular calcium levels. A decrease in cardiac muscle contractility has been shown after exposing cardiac myocytes to TNFα as well as IL-1β and IL-6[40,41], but the mechanism is not fully understood[42].

Organ perfusion pressure depends on the contraction or relaxation of arterioles. The degree of contraction of smooth muscle in the arterial walls, referred to as vascular tone, depends on intracellular calcium concentration. This is in turn regulated by intrinsic and extrinsic factors. Examples of intrinsic factors are endothelial secretions, or NO and endothelin, and vasoactive metabolites, e.g. acidosis and hypoxia. Extrinsic regulation consists of sympathetic regulation and hormones including adrenaline, angiotensin II and vasopressin[37]. Inflammation affects intrinsic as well as extrinsic regulation. Cytokines and PAMPs induce synthesis of inducible NO[43] and endothelin-1 triggers release of IL-6 and 2[44] whereas downregulation of receptors have been shown in sepsis for adrenaline[45] and vasopressin[46].

Vascular permeability and oedema

The forces acting on plasma fluid transport across vascular endothelium are classically described by the Starling equation[47]:

$$J_v = L_p S((P_c - P_i) - \sigma(\pi_p - \pi_i))$$

Where there are two forces acting to keep fluid in the vessel, interstitial hydrostatic pressure (P_i) and plasma protein oncotic pressure (π_p) and two forces acting to move fluid across the endothelium, capillary hydrostatic pressure (P_c) and interstitial oncotic pressure (π_i). The net driving force is multiplied by the surface area for filtration (S) and the conductivity of the endothelium (L_p) giving us the trans endothelial solvent filtration volume.

The reflection coefficient (σ) corrects the actual colloid osmotic pressure difference to the observed or effective pressure.

Since the discovery of the glycocalyx lining of the endothelium, the equation has been somewhat modified in that the oncotic pressure of the interstitium has been replaced by oncotic pressure in the sub-glycocalyx space[48]. In this context, σ can be thought of as indicative of the integrity of the glycocalyx. If the glycocalyx is intact, σ is close to 1.

In the healthy situation, there is a filtration of fluid over the endothelium that is transported away by the lymphatic system and returned to the circulation. A change in any of the variables of the Starling equation which is big enough to result in fluid transport over the epithelium larger than can be removed by the lymphatic system will result in oedema formation.

Oedema formation increases the diffusion distance for oxygen and nutrients, possibly affecting cellular metabolism. In the same way, diffusional removal of potentially toxic by-products of cellular metabolism is impaired.

Inflammation affects several of the factors of the Starling equation. Vascular permeability is increased by several mechanisms, HBP being one. These lead to increased flow but also allow passage of proteins to the interstitium, resulting in decreased osmotic pressure gradient. Capillary vasodilatation results in an increase in the filtration surface. Taken together, this can rapidly result in oedema formation. Oedema is a very common clinical feature of critical illness, regardless of the type of medical or surgical inciting problem.

HBP and circulatory failure

An inflammatory reaction increasing the vascular permeability of large enough areas of capillaries might cause circulatory compromise due to loss of circulating blood volume. This could be one possible mechanism by which HBP may be associated with circulatory insufficiency. A number of studies have investigated aspects of HBP concentration in plasma and compromised circulation.

For patients with suspected infection in the emergency department, plasma concentration of HBP has been shown to perform well predicting development of circulatory instability[49,50]. For patients cared for in intensive care units, elevated plasma concentration of HBP has been found in patients with shock but not differentiating septis from other causes[51]. In trauma patients, HBP concentration is higher in those presenting with shock[52], and after cardiac arrest higher HBP concentration is associated with increased need for circulatory support[53,54]. Patients with burns have initial high plasma concentrations of HBP corresponding to the high vascular permeability phase[55]. In patients with sepsis, a weak correlation has been found between plasma concentration of HBP and fluid overload[12] (Table 1). Recently, increased concentrations have been described in patients with myocardial infarction[56].

Table 1. Data on HBP concentrations and circulatory instability

Study	Patient group	Timing of sampling	Concentration of HBP (ng/ml)	Conclusion
Johansson, Lindbom et al. 2009	ICU after burn	Day 1	24(19-28) (mean+95% CI)	Increase correlated with time of increased vascular permeability
Linder, Christensson et al. 2009	Patient with suspected infection and fever	ED	Cut of >15	ROC AUC 0.95(CI 0.92-0.98) predicting hypotension
Chew, Linder et al. 2012	ICU patients in shock	6h after onset of shock	24.1(9.8-125.7) vs 27.2(9.0-122.2) (median+range)	No difference sepsis vs no sepsis as cause of shock
Dankiewicz, Linder et al. 2013	ICU after cardiac arrest	12h after cardiac arrest	11.8(9.1-21.6) (median+IQR)	Higher if circ.subscore of SOFA ≥ 4 (p=0.03)
Linder, Arnold et al. 2015	Patient with suspected infection and fever	ED	63.5(35.1-114.1) vs 18.8(10.6-29.7) (median+IQR)	Higher in those developing organ failure
Bentzer, Fisher et al. 2016	Patients with septic shock in ICU	After 6h of shock	25(8-71) (median+IQR)	Weak correlation with fluid overload (rho 0.13, p=0.01), Correlates with dose of noradrenaline
Ristagno, Masson et al. 2016	ICU after cardiac arrest	Admission to ICU	Approx. 20 vs 12 (median)	Higher if circ.subscore of SOFA ≥ 4 (p<0.01)
Halldorsdottir, Eriksson et al. 2018	ICU after trauma	Median 17 h after trauma	38.2(29.2-47.2) (mean+95% CI)	Higher if shock (p<0.05)

Acute respiratory distress syndrome

The acute respiratory distress syndrome (ARDS), first described in 1967[57], is characterised by non-hydrostatic pulmonary oedema after a clinical insult leading to impaired oxygenation. Recognised insults that can result in ARDS can be pulmonary, e.g. pneumonia or aspiration, as well as extra pulmonary, e.g. trauma, pancreatitis or sepsis.

Since 1967 several different classification systems have been used to diagnose and grade ARDS, the most recent being the Berlin definition (table 2)[58].

In previous definitions, the term acute lung injury (ALI) was used for the mildest form of ARDS.

The pathophysiology behind the development of ARDS is not fully understood, but is described as occuring in three phases: acute, subacute and chronic. In the acute phase, the most prominent feature is increased vascular permeability of pulmonary vasculature causing interstitial oedema and alveolar flooding. While leukocytes, especially macrophages and neutrophils, are main contributors to endothelial and epithelial injury, intravascular and alveolar coagulation is also prominent (Fig.2).

Subsequently, hyaline membranes develop in the alveoli. In the subacute phase, there is reabsorption of the oedema, proliferation of alveolar epithelial type II cells, and infiltration of fibroblasts with deposition of collagen fibres. Finally, in the chronic stage, there is repair, clearance of the neutrophils, and some degree of fibrosis may remain[59,60].

There is a wide variety of described incidences of ARDS, ranging from 10 (in South America) to 80 (in the USA) per 100000 person years. This disparity likely reflects varying medical resources in different regions and difficulties in setting the diagnosis, though an actual difference in incidence between populations is possible[59]. While it seems like mortality in ARDS is decreasing over time, it is still reported being as high as 45% in the group with severe ARDS[58].

Table2. The Berlin Definition of Acute Respiratory Distress Syndrome

Timing	Within 1 week of a known clinical insult or new or worsening respiratory symptoms
Chest imaging	Bilateral opacities— not fully explained by effusions, lobar/lung collapse, or nodules
Origin of oedema	Respiratory failure not fully explained by cardiac failure or fluid overload. Need objective assessment (e.g., echocardiography) to exclude hydrostatic oedema if no risk factor present
Oxygenation	
Mild	PaO_2:FiO_2 < 39.9 KPa with CPAP or PEEP ≥ 5
Moderate	PaO_2:FiO_2 < 26.6 KPa with PEEP ≥ 5
Severe	PaO_2:FiO_2 < 13.3 KPa with PEEP ≥ 5

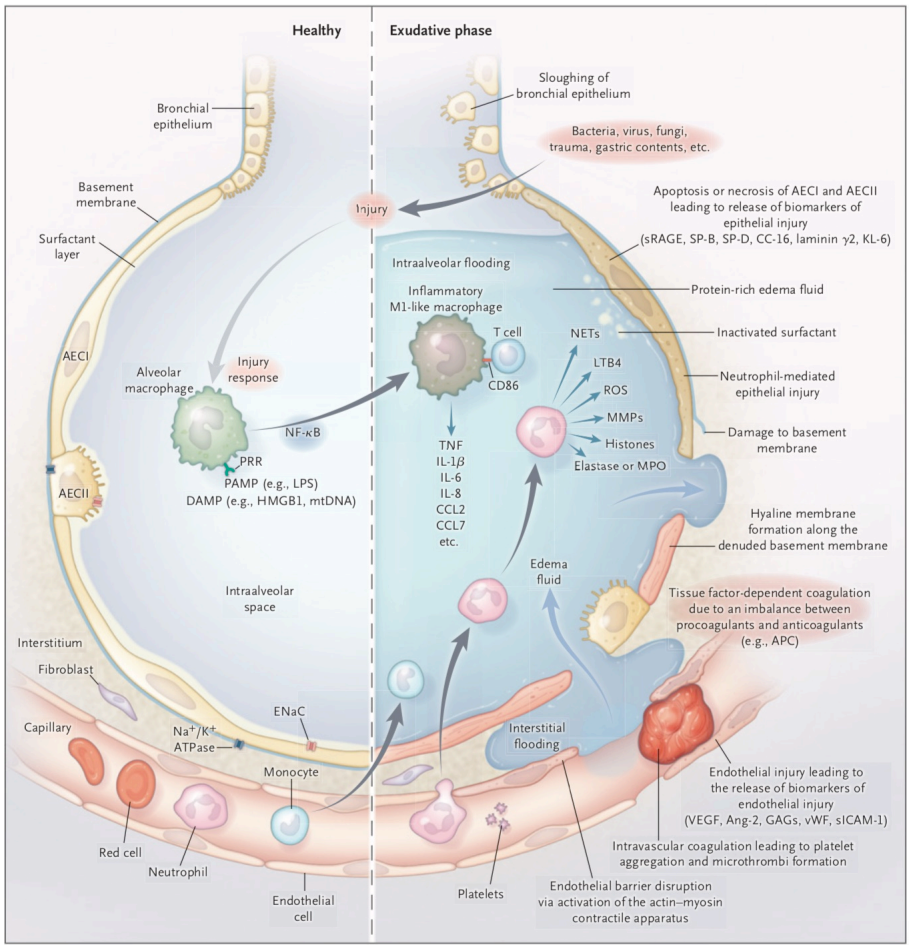

Figure 2. Schematic overview of the acute phase of ARDS. Reproduced with permission from Thompson BT, Drazen JM, Chambers RC, Liu KD. Acute Respiratory Distress Syndrome. New England Journal of Medicine 2017; 377: 562-72, Copyright Massachusetts Medical Society.

HBP and respiratory failure

Given the contribution of increased vascular permeability to respiratory dysfunction, the relationship between plasma concentration of HBP and respiratory insufficiency has been the focus of a number of previous studies (Table 3).

Patients with acute lung injury have an elevated HBP plasma concentration[61]. In patients with H_1N_1 influenza, plasma concentration of HBP correlates with decreased oxygenation[62]. In trauma patients, HBP concentration is higher in those developing acute respiratory distress syndrome[63]. Plasma concentrations of HBP are higher in patients with ARDS than patients with hydrostatic pulmonary oedema[64].

Another recent study has shown a correlation between decreased oxygenation and HBP concentration in ICU patients with sepsis, as well as demonstrating histological lung injury in mice after intravenous injection of HBP[12]. Endotracheal instillation of HBP in mice also produces histological lung injury and oedema[65].

Table 3. Data on concentrations of HBP and respiratory failure

Study	Patient group	Timing of sampling	Concentration of HBP (ng/ml)	Conclusion
McAuley, O'Kane et al. 2013	Intubated patients in ICU with ALI	Not available	16.7 (median)	Levels higher than in healthy controls ($p < 0.01$)
Johansson, Brattstrom et al. 2013	ICU after trauma	Within 36 h	12.3(8.6-16.0) vs 7.4(4.0-9.4) (median+IQR)	Higher levels in patients developing ARDS ($p=0.026$) ROC AUC 0.748
Kaukonen, Linko et al. 2013	ICU with H_1N_1 influenzae	Day 2 in ICU	188(86-389) vs 81(55-112) (median+IQR)	Higher levels if PF ratio < 100 mmHg ($p < 0.05$). Correlates with lowest P:F ratio (rho 0.26, $p=0.08$)
Lin, Shen et al. 2013	ICU with respiratory failure	Not available	17.5(12.0-24.1) vs 9.5(7.98-12.2) (median+IQR)	Higher in ARDS compared to congestive heart failure ($p<0.001$)
Johansson, Steinvall et al. 2015	Patients in burn ICU	Within 24 h in ICU	25.9(13.8-30.8) vs 20.6(17.6-43.8) (median+IQR)	No correlation with increase in pulmonary vascular permeability ($p=0.31$) or decrease in P:F ratio ($p=1.0$)
Bentzer, Fisher et al. 2016	Patients with septic shock in ICU	After 6h of shock	47(23-103) vs 22(7-62) (median+IQR)	Higher levels if P:F ratio<100 mmHg ($p < 0.01$). Correlates with lowest P:F ratio (rho -0.25, $p<0.001$)

Ventilator induced lung injury

When respiratory compromise becomes severe enough, intubation and mechanical ventilation may be lifesaving. However, mechanical ventilation can by itself cause injury to the lungs[66]. The mechanisms of injury are classically divided into four categories: barotrauma, volutrauma, atelectrauma and biotrauma[67]. Barotrauma is lung injury caused by high transpulmonary pressure. This may occur even at low airway pressure if pleural pressure is extremely negative (e.g. forceful inspiratory effort). In volutrauma the lung injury is caused by alveolar overdistension.

Atelectrauma causes lung injury by cyclic opening and collapse of atelectatic but recruitable lung units.

Trying to minimize additional injury caused by ventilation is a daily challenge in caring for ICU patients. Not exceeding tidal volumes of 6 ml/kg or plateau pressures of 30 cm H_2O have been shown to reduce mortality in acute respiratory distress syndrome patients[68] presumably by reducing baro- and volutrauma. Positive end expiratory pressure is commonly used to prevent atelectasis and reduce atelectrauma, but evidence of improved outcomes is not as robust for this intervention[67].

Apart from lung injury caused by mechanical forces, the injury is also aggravated by the immunological response to the mechano-trauma, which is referred to as biotrauma[69]. Biotrauma is largely dependent on leukocyte function demonstrated by apparent resistance to injurious ventilation in leukocyte-depleted rabbits[70]. The mechanism by which leukocytes aggravate the lung injury is not fully understood, but IL-6 and TNFα are thought to be involved,[66] and increased vascular permeability and alveolar flooding is a prominent feature[69].

HBP and renal failure

Historically, acute kidney injury (AKI) associated with critical illness was believed to be caused by decreased renal blood flow due to low cardiac output and/or hypotension causing ischemia and tubular necrosis. Over the last decade this theory has been questioned, especially in AKI associated with sepsis[71]. In animal models of septic AKI, renal blood flow is increased[72] and histological examination of renal biopsies show limited cell damage[73]. At present, the cause of AKI is believed to be a combination of inflammation, cell cycle arrest, and microvascular alterations, including increased vascular permeability[74].

Apart from Paper III in this thesis, there are two other studies investigating plasma concentrations of HBP and development of AKI in humans. One published just before and one just after our study. In the first, HBP concentration is shown to be associated with and predict AKI in patients with sepsis. In the same paper, infusion of HBP in mice induces tubular inflammation and signs of renal oedema[75]. In the second study, adding HBP concentration to a known risk stratification tool for development of AKI in sepsis patients is shown to increase the performance of the tool in predicting AKI[76].

Findings in a mouse model of septic AKI suggest that HBP is important for activation of M1 macrophages in the kidneys[77] further supporting the hypothesis that HBP and inflammation are important contributors in the development of kidney injury during sepsis.

Renal clearance

Renal clearance is defined as the theoretical volume of plasma completely cleared from a substance over time. Under the condition that the plasma

concentration of the substance is constant, renal clearance can be calculated as follows:

$$K = \frac{CU \times Q}{CB}$$

where

K is the clearance (ml/min)

CU is the concentration in urine

Q is the urine flow

CB is the concentration in plasma

It should be noted that the formula is a way to describe the amount of a substance excreted in urine over time in relation to the plasma concentration. It does not say anything about interactions between filtration, tubular reabsorption or active secretion. Another possible confounder is if the substance of interest is produced in the urine which could lead to an overestimation of clearance.

There are studies on HBP concentration in urine in the context of pyelonephritis, uncomplicated urinary tract infections, and asymptomatic bacteruria[78-80]. The source of this HBP is probably leukocytes in urine due to local infection. There are no published data to date on concentrations of HBP in urine related to plasma in the absence of urinary tract infections.

Other small molecular weight proteins roughly the same size as HBP are often filtrated in the glomeruli and reabsorbed in the tubuli[81]. The presense of HBP in urine of healthy controls in the studies on urinary tract infections suggests that HBP may, in part, be eliminated via urine.

Aims

Paper I

The aim was to determine if plasma concentration of HBP sampled on admission to intensive care is associated with the development of circulatory or respiratory failure in the following 3 days in a critically ill population with mixed diagnoses.

Paper II

The aim was to test if plasma concentration of HBP sampled on admission to intensive care is associated with the development of acute kidney injury in the following seven days in a critically ill population with mixed diagnoses.

Paper III

The aim of was to assess concentration of HBP in plasma and bronchoalveolar lavage fluid (BALF) in a pig model subjected to ventilator induced lung injury (VILI). A further aim was to compare concentrations of HBP in BALF from healthy individuals and intubated ICU patients receiving protective ventilation

Paper IV

The aim was to compare renal clearance of HBP in healthy individuals and in burn ICU patients with normal and impaired kidney function. An additional aim was to to assess excretion of HBP in ICU patients treated with continuous renal replacement therapy.

Materials and Methods

Scoring systems

Two different systems for scoring severity of illness and one for scoring acute kidney injury are used in this thesis.

Simplified acute physiology score 3

Simplified acute physiology score 3 (SAPS 3) is used to predict mortality[82]. It is calculated on admission to intensive care based on factors before admission, comorbidities, reason for admission, and physiology at admission (Table 4). For accurate mortality prediction, SAPS 3 needs to be calibrated over time and for specific regions.

Table 4. SAPS 3 score

Pre-admission	Age	Comorbidity	Haematological cancer
	Length of stay		Chronic heart failure
	Location in hospital		Cancer therapy
	Use of vasopressors		Cirrhosis
Admission	Planned/Unplanned		AIDS
	Cardiovascular		Cancer
	Hepatic	Physiology	Glasgow Coma Scale
	Digestive		Bilirubin
	Neurologic		Creatinine
	Surgery planned/acute		Leucocytes
	Type of surgery		pH
	Type of infection		Platelets
			Temperature
			Blood pressure
			Oxygenation

Sequential organ failure assessment score

The sequential organ failure assessment (SOFA) score was initially designed to evaluate progression of organ failure in patients with sepsis. It has later been found to be useful for predicting mortality in ICU patients with mixed diagnoses[83]. It is calculated daily using the worst values of six organ systems and assigning them with a score of 0-4 (Table 5).

Table 5. SOFA score

	0	1	2	3	4
Oxygenation (PaO_2:FiO_2)	>53	≤53	≤40	≤27	≤13
Coagulation (Platelets x 10^9/l)	>150	≤150	≤100	≤50	≤20
Hepatic (Bilirubin(umol/l))	<20	20-32	33-101	102-204	>204
Cardiovascular *=μg/kg/min	MAP≥70	MAP<70	Dopamine ≤5* Dobutamine	Dopamine>5* A/NA≤0.1*	Dopamine>15* A/NA>0.1* Levosimendan Vasopressin
CNS (GCS)	15	13-14	10-12	6-9	3-5
Renal (Creatinine(μmol/l)) (Diuresis(ml/24h))	<110	110-170	171-299	300-440 <500	>440 <200

Acute kidney injury stage

The presence and severity of acute kidney injury (AKI) is scored with AKI criteria according to "Kidney Disease: Improving Global Outcomes" (KDIGO) guidelines[84] (Table 6).

The creatinine elevation or urine output resulting in the highest AKI stage is normally used. In Paper III, acute kidney injury is scored only with the creatinine part of the staging system due to uncertain registrations of urine output prior to admission to ICU.

Table 6. Acute kidney injury stage

Stage	Serum creatinine	Urine output
1	1.5-1.9 times baseline OR ≥26.5 mmol increase	<0.5 ml/kg/h for 6-12 hours
2	2.0-2.9 times baseline	<0.5ml/kg/h for ≥ 12hours
3	3.0 times baseline OR increase to ≥353.6 mmol/l	<0.3 ml/kg/h for ≥ 24 hours

Papers I + II

Papers I and II are based on an observational study in the 8-bed mixed ICU of Östersund hospital, a 300-bed hospital in Sweden. All patients admitted to the ICU between 1 February 2012 and 31 January 2013 were screened for inclusion. Inclusion criteria were admission to the ICU and presence of, or need for an arterial catheter to be inserted. Patients under the age of 18 years and those transferred from other ICUs were excluded. Blood samples for HBP analyses were collected on admission to the ICU and on the two following days.

Simplified acute physiology scores 3 were recorded on admission. Sequential organ failure assessment scores were also recorded in parallel and then day 1 and day 2. Final diagnosis was set retrospectively by chart review. Severe sepsis was defined as suspected or documented infection, two or more systemic inflammatory response syndrome (SIRS) criteria and organ dysfunction[85].

Paper I

The SOFA score was used to classify the patients as having or not having organ failure. The measurement on day 2 corresponded to a value most often 48-72 hours after admission to the ICU. If a patient was discharged, alive or dead, before that registration, the last available registration was used. The main outcome variables were respiratory and circulatory failure. The outcome variable circulatory failure was defined as a SOFA circulatory sub score of 4. The outcome variable respiratory failure was defined as a P:F ratio (arterial partial pressure of oxygen/fraction of insipired oxygen) < 27 (The oxygenation cut off between mild and moderate in the Berlin ARDS classification[58]).

Paper II

Serum creatinine concentration was recorded on admission and on the following 6 days. A pre-admission, habitual concentration of creatinine was sought in each patient's chart. If a habitual concentration could not be found, this was recorded as creatinine corresponding to a glomerular filtration rate (GFR) of 75ml/min/m^2 using the MDRD (modification of diet in renal disease) formula as recommended by the Acute Dialysis Quality Initiative[86]. The MDRD formula was also used to calculate estimated GFR on admission. The presence and severity of AKI was graded daily during the first 7 days of the ICU stay using the AKI criteria according to Kidney Disease: Improving Global Outcomes (KDIGO) guidelines[84]. As reliable data on urine output were missing, the AKI stage was calculated using creatinine elevation criteria only. Patients with AKI stage 2 or 3 at admission were excluded. The highest AKI stage during the first 7 days was recorded for each patient. In the event of discharge (alive or dead) from ICU prior to 7 days, the highest AKI stage during the ICU stay was used.

Paper III

Animal studies

Twelve juvenile Yorkshire/Swedish landrace pigs were anesthetised. To render the animals' lungs more susceptible to VILI, surfactant depletion was achieved by saline lavage in all animals. Immediately after surfactant depletion, the animals were randomized to either protective or injurious ventilation. In the group with protective ventilation, tidal volumes were kept at 8 ml/kg, PEEP was elevated to 8 cm H_20 and FiO2 adjusted to achieve normoxia. The group with injurious ventilation (referred to as the VILI group) received tidal volumes of 20 ml/kg, zero end expiratory pressure, and FiO_2 1.0. The animals were ventilated in either fashion for 6 hours, and then euthanized by injection of potassium chloride under continuous deep anaesthesia. Samples of blood and BALF (broncho-alveolar lavage fluid) were collected before surfactant depletion and after 1, 2, 4, and 6 hours of ventilation (Figure 3).

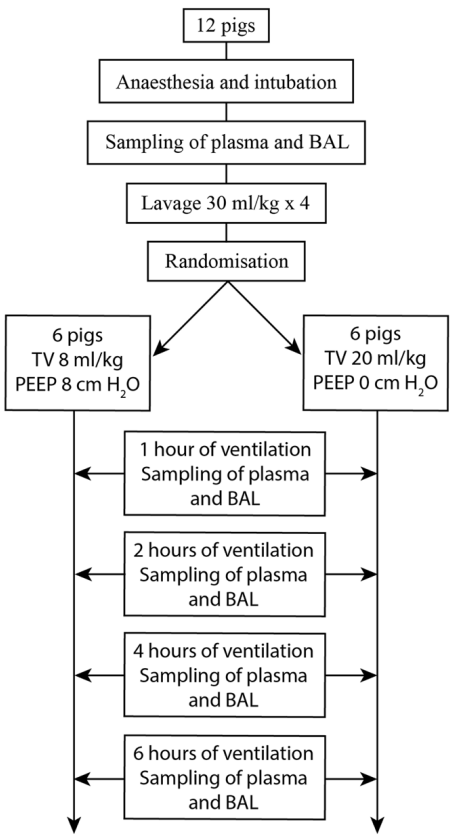

Figure 3. Schematic overview of the protocol. BAL: bronchoalveolar lavage. TV: tidal volume. PEEP: positive end expiratory pressure. Tyden J, Larsson N, Lehtipalo S, Herwald H, Hultin M, Wallden J, Behndig AF, Johansson J, (2018) Heparin-binding protein in ventilator-induced lung injury. Intensive Care Med Exp 6: 33.

BALF was collected using a flexible fiberoptic bronchoscope. The bronchoscope was inserted through the tracheal tube and carefully wedged into a bronchus. As sampling was repeated, care was taken to avoid multiple samples from the same location. Three aliquots of 50 ml sterile saline were infused and gently suctioned back after each infusion.

Human studies

Healthy volunteers

Sixteen healthy, non-smoking subjects were recruited to the study. The subjects received 1 mg atropine subcutaneously as pre-medication. Lidocaine was used for topical anaesthesia. Bronchoscopy was performed using a flexible video bronchoscope introduced through the mouth. Bronchoalveolar lavage with 3 × 60 ml of saline solution from the middle or lingula lobes was performed.

ICU patients

Ten patients older than 18 years of age admitted to the ICU of Östersund Hospital and intubated during the previous 24-hour period were included.

The SAPS 3 at admission to the ICU and the highest respiratory pressure settings from intubation to sampling were recorded. Plasma concentration of HBP was sampled at the time of intubation. Diagnosis was determined retrospectively through chart review.

BALF was collected using a flexible fiberoptic bronchoscope. The bronchoscope was inserted through the tracheal tube and wedged into a bronchus of the middle or lingual lobe. If lung pathology was unilateral, the contralateral side was used. A total volume of 50 ml of sterile saline was instilled and suctioned back four times. All bronchoalveolar lavage fluid obtained was filtered, centrifuged, and the supernatant was frozen at −80°C until analysis.

Paper IV

Healthy volunteers

The study entailed 8 healthy volunteers. Inclusion criteria were self-reported health and a negative urinary dipstick. Urine was collected throughout the 8-hour study period. Urine volume was measured, and a urine sample drawn from the collected urine volume. To verify that plasma concentrations of HBP were stable, 8 blood samples were drawn hourly from one volunteer, 4 samples were drawn from two volunteers and from the other five volunteers 2 blood samples were drawn, one in the morning and one in the afternoon. For each participant mean plasma concentration of HBP was used for calculating clearance.

Burn ICU patients

The study was conducted at a national burn treatment centre (Linköping University Hospital Burn Unit) in a cohort of patients previously reported[87]. Twenty patients with burns involving 20% or more of the total body surface area (TBSA%) were included. Patients were treated in accordance with a standardized protocol [88], including the Parkland formula for early resuscitation, ventilator treatment when needed, early enteral nutrition, and early excision and grafting of the burn wound, starting within 1-2 days.

Plasma and urine samples were collected and registration of urine flow was documented twice the first day and once a day on the third, the sixth and the eighth day after arrival to hospital. HBP and Cystatin C concentrations were analysed in the plasma samples. HBP concentration was analysed in the urine samples.

Cystatin C is emerging as a potential marker of impaired renal function in burn patients[89,90]. The concentration of the upper reference interval (1.44 mg/L) was used as cut off for impaired renal function.

CRRT patients

The study was an observational study in the 8-bed mixed ICU of Östersund hospital. During the period 2012-2016, we included 32 patients over 18 years who started CRRT in the ICU. Simplified acute physiology scores 3 were recorded on admission. The CRRT equipment used was the Baxter/Gambro Prisma™. Continuous veno-venous hemodiafiltration (CVVHDF) was performed with a hollow fibre haemofilter, AN69, either ST150™ or Oxiris™. Citrate was used for anticoagulation. All patients underwent continuous veno-venous hemodiafiltration with an effluent flow of 30-35 ml/kg/h and a blood flow of 160-200 ml/min.

Plasma samples were drawn when CRRT was started and at the time of changing the first and second drainage bag. Samples from the second drainage bag were collected and total volume in the drainage bags was measured.

Analysis of HBP

Concentrations of HBP in the samples were measured with an enzyme-linked immunosorbent assay (ELISA) as described previously[6]. In brief, this is a sandwich ELISA where plates are coated with a mouse monoclonal antibody against HBP. After adding the study samples, along with samples with known concentrations of HBP, a polyclonal rabbit antiserum against HBP was added. For the detection of antibodies bound to HBP, a peroxidase-conjugated antibody against rabbit immunglobulin G was added, and the optical density was determined at 420 nm. The technique has an intra- and interassay variability of less than 10%.

Statistics

Descriptive data are presented as medians with interquartile ranges or means with 95% confidence intervals.

In Papers I and II, plasma concentrations of HBP were log-transformed for statistical testing because of skewed distribution of data.

To compare the significance of differences between groups, we used Student's *t* test or the Mann–Whitney U test, as appropriate. The significances of differences between categorical variables were evaluated using Fisher's exact test. Significance of difference between related groups of non-parametric data was evaluated with Wilcoxon signed rank test, in Papers III and IV.

Comparison of HBP concentrations between multiple groups of patients in Papers I and II, was made by one-way ANOVA with a post hoc analysis with compensation for multiple comparisons according to Bonferroni.

In Paper II, logistic regression was used to calculate odds ratios for HBP concentration, SAPS 3, sex, age, CRP, estimated GFR and WBC in explaining the development of renal failure. The parameters were analysed in univariate models and significant parameters were included in a multivariate model. Non-significant parameters were then excluded to reach a final multivariate model.

ROC curves were used to analyse the discriminatory capacity of HBP concentration on circulatory, respiratory and renal failure in Papers I and II.

In Paper IV, Spearman's non-parametric correlation coefficient was used to assess correlation between HBP concentrations in plasma and effluent fluid of CRRT.

Probabilities of less than 0.05 were accepted as significant for identifying statistical differences in grouped comparisons. The data were analysed using SPSS (IBM SPSS Statistics for Macintosh, Version 23.0, Armonk, NY: IBM Corp) or Statistica 12 (StatSoft™ Inc, Tulsa, OK, USA).

Ethics

Paper I + II

The Regional ethics review board in Linköping gave ethical approval (D.nr. 2010/427-31, 30/9 2011). Verbal consent was given by the patient or next of kin, if the patient was not able.

Paper III

Animal studies

Ethical permission from the Umeå Animal Experimental Ethics Committee (D.nr. A43-12, 28/3 2012). All procedures were carried out in accordance with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals and the Guide for the Care and Use of Laboratory Animals (1996) prepared by the National Academy of Sciences' Institute for Laboratory Animal Research.

Healthy volunteers

The study was approved by the Umeå University Ethics Committee (D.nr. 2018-30-32M, 26/4 2018). All subjects gave their written informed consent.

ICU patients

The regional ethics review board in Linköping gave ethical approval (D.nr.2010/427-31, 30/9 2011). Oral consent was given by next of kin.

Paper IV

Healthy volunteers

The Regional Ethics Review Board in Umeå approved the study (D.nr. 2015/474-31, 8/2 2016). All subjects gave their written informed consent.

Burn patients

The Regional Ethics Review Board in Linköping approved the study (D.nr. M210-08, 9/1 2009). Consent was given by the patient or next of kin if the patient was not able.

CRRT patients

The Regional Ethics Review Board in Linköping approved the study (D.nr. 2010/427-31, 30/9 2011). Consent was given by the patient or next of kin, if the patient was not able.

Results

Paper I

Of 589 consecutive patients admitted to the ICU during the study period, 329 were eligible for inclusion, and 278 were included. Details of patients, type of admissions, and outcome are shown in Table 7.

Table 7. Details of patients.

Age (years)	68 (54-76)
Sex (Male/female)	169/109
log HBP(ng/ml) (mean, 95% CI)	3.79 (3.67-3.90)
HBP (ng/ml)	36.6 (24.5-63.3)
CRP (mg/L) (mean, 95% CI)	83.3 (68.5-98.0)
WBC (x109/L) (mean, 95% CI)	13.7 (12.6-14.7)
Main diagnosis (n, %)	
Sepsis	83 (30%)
Trauma	34 (12%)
Intoxication	11 (4%)
Cardiac arrest	18 (6%)
Gastrointestinal bleeding	11 (4%)
Other surgical	28 (10%)
Other medical	93 (33%)
SAPS 3 score	58 (48-70)
Maximal SOFA score	6 (4-9.5)
ARDS (n, %)	33 (12%)
Duration of stay in ICU (days)	2 (1-3)
ICU mortality (n, %)	33 (12%)
30-day mortality (n, %)	64 (23%)

Data presented as median (interquartile range) where not otherwise indicated or %. ARDS (acute respiratory distress syndrome); HBP (Heparin-binding protein); CRP (C-reactive protein); WBC (white blood count); SAPS (simplified acute physiology score); SOFA (sequential organ failure assessment); ICU (intensive care unit). Tyden J, Herwald H, Sjoberg F, Johansson J. Increased Plasma Levels of Heparin-Binding Protein on Admission to Intensive Care Are Associated with Respiratory and Circulatory Failure. PLoS One. 2016;11(3):e0152035. Epub 2016/03/24. doi: 10.1371/journal.pone.0152035. PubMed PMID: 27007333; PubMed Central PMCID: PMCPMC4805239.

There was an association between high plasma concentration of HBP on admission and a P:F-ratio of less than 27 kPa at the last registration ($p<0.001$) (Fig.4). There was an association between HBP-concentration on admission and circulatory SOFA sub score of 4 at the last registration ($p<0.001$) (Fig.5). There was also an association between plasma concentration of HBP on admission and 30-day mortality ($p=0.002$).

ROC-curves using sensitivity and specificity of plasma concentration of HBP at admission at different diagnostic cut-off levels showed areas under the curve of 0.61 (0.54-0.69) for predicting a P:F ratio less than 27 kPa at last registration, 0.65 (0.54-0.76) for a circulatory sub score of SOFA of 4 at last registration and 0.64 (0.56-0.72) for 30-day mortality.

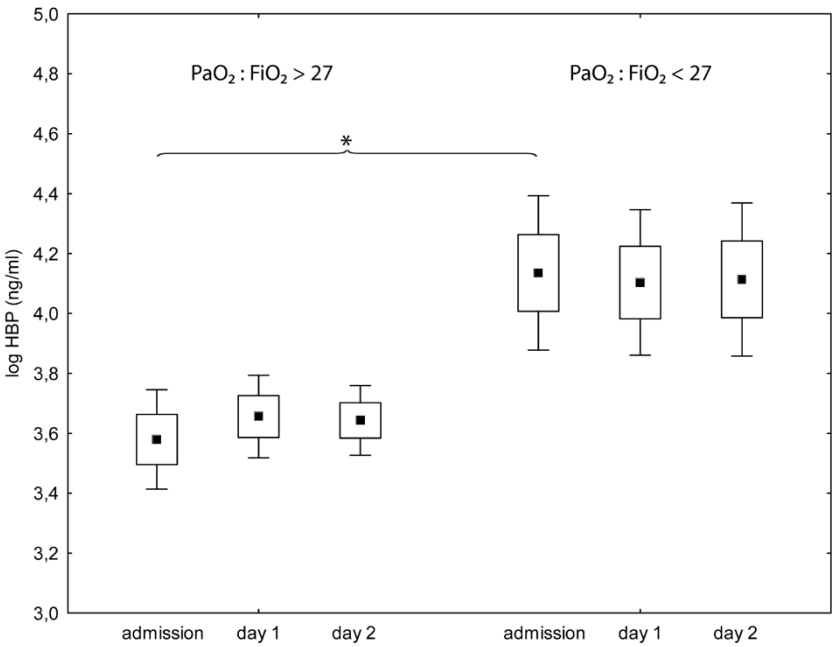

Figure 4. Plasma concentration of heparin binding protein on admission to intensive care and the following two days for patients with an arterial PaO_2/Fraction of inspired O_2 of more than 27 kPa (left) or less than 27 kPa (right) on the last registration. Values at admission are higher in patients with PaO_2/Fraction of inspired O_2 < 27 ($p<0.001$). Square, box and bracket indicates mean, standard deviation and 95% confidence interval. * = statistical difference between groups $p<0.05$. Tyden J, Herwald H, Sjoberg F, Johansson J. Increased Plasma Levels of Heparin-Binding Protein on Admission to Intensive Care Are Associated with Respiratory and Circulatory Failure. PLoS One. 2016;11(3):e0152035. Epub 2016/03/24. doi: 10.1371/journal.pone.0152035. PubMed PMID: 27007333; PubMed Central PMCID: PMCPMC4805239.

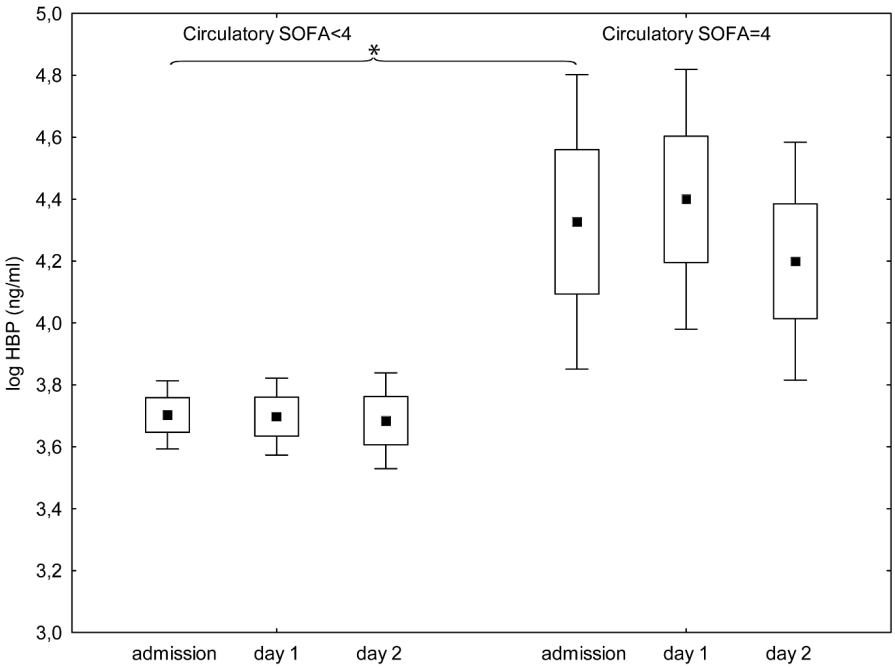

Figure 5. Plasma concentration of Heparin binding protein on admission to intensive care and the following two days for patients with circulatory sub score of SOFA less than 4 (left) or 4 (right) on the last registration. Circulatory SOFA indicates circulatory sub score of Sequential organ failure assessment. Values at admission are higher in patients with a circulatory sub score of 4 ($p<0.001$). Square, box and bracket indicates mean, standard deviation and 95% confidence interval. * = statistical difference between groups $p<0.05$. Tyden J, Herwald H, Sjoberg F, Johansson J. Increased Plasma Levels of Heparin-Binding Protein on Admission to Intensive Care Are Associated with Respiratory and Circulatory Failure. PLoS One. 2016;11(3):e0152035. Epub 2016/03/24. doi: 10.1371/journal.pone.0152035. PubMed PMID: 27007333; PubMed Central PMCID: PMCPMC4805239.

Paper II

Out of 589 patients admitted to the ICU during the study period, 245 were included and 59 of these fulfilled the criteria for severe sepsis.

Plasma log concentration of HBP (ng/ml) in the groups developing different stages of AKI within the first 7 days were as follows: AKI stage 0 3.5 (3.4-3.7) (mean, 95% CI), AKI stage 1 3.7 (3.5-4.0), AKI stage 2 4.4 (3.5-4.8), AKI stage 3 4.6 (3.8-5.2) (Fig.5). Plasma concentration of HBP were higher in patients with AKI stage 3 than in those with AKI stage 0 ($p<0.01$) and AKI stage 1 ($p=0.04$), but not higher than in those with AKI stage 2 ($p=0.67$) (Fig.6).

Odds ratios from univariate logistic regression using the outcome AKI stage 2-3 as opposed to AKI stage 0-1 for SAPS 3, HBP, CRP, WBC, age, estimated GFR and sex are shown in Table 8. In the final adjusted model, the odds ratio for the concentration of HBP contributing to the development of AKI stage 2-3 was 1.76 (95% CI 1.10-2.98, p=0.02) (Table 8).

The discrimination capacity, presented as an area under the curve (AUC) for a receiver operating characteristics curve, on AKI stage 3 was 0.68 (95% CI 0.54-0.83) for the HBP concentration at admission. Looking only at patients with severe sepsis (n=59), the AUC for AKI stage 3 was 0.93 (95% CI 0.85-1.00) for the HBP concentration at admission.

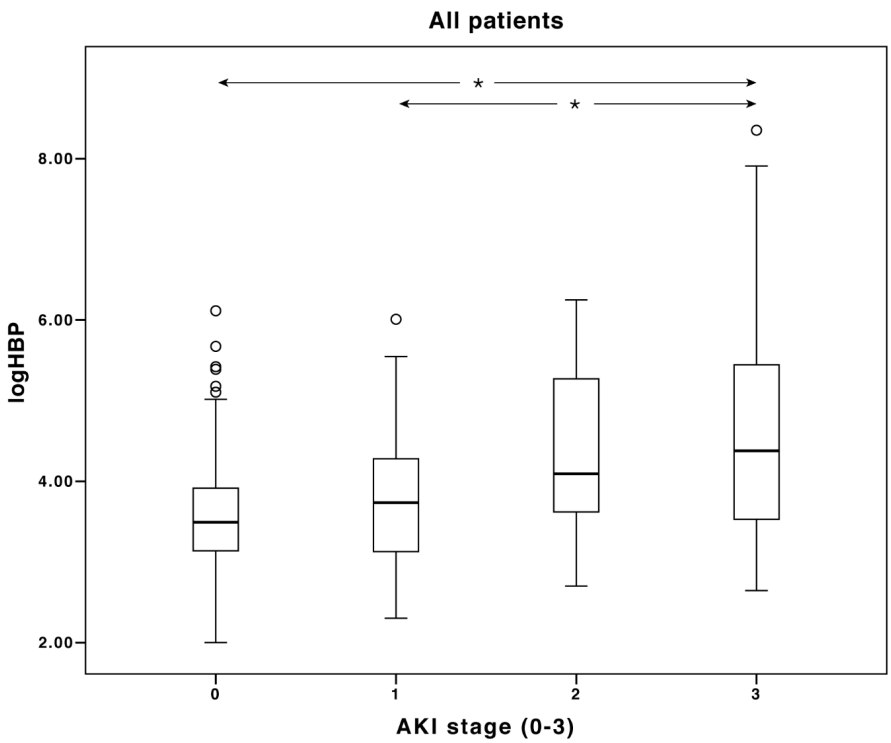

Figure 6. Plasma concentration of heparin-binding protein in the groups of acute kidney injury (AKI stage 0-3) for all patients. Boxes indicate second to third quartile with mean. Brackets indicate min-max values. Circles indicate outliers. Stars indicate differences for paired comparisons ($p<0.05$). Tyden J, Herwald H, Hultin M, Wallden J, Johansson J. Heparin-binding protein as a biomarker of acute kidney injury in critical illness. Acta Anaesthesiol Scand 2017; 61: 797-803. With permission from John Wiley and sons.

Table 8. Logistic regression for development of AKI stage 2-3.

	Univariate Odds ratio (95% CI)	p	Final multivariate model Odds ratio (95% CI)	p
HBP(log)	2.38 (1.50-3.76)	<0.01	1.76 (1.10-2.98)	0.02
eGFRadm ($mL/min/m^2$)	0.97 (0.96-0.99)	<0.01	0.98 (0.96-0.99)	<0.01
Male sex	3.73 (1.24-11.23)	0.02	3.42 (1.08-10.88)	0.04
SAPS3	1.06 (1.03-1.09)	<0.01		
WBC ($x10^9/L$)	1.06 (0.99-1.11)	0.07		
Age (years)	1.03 (1.00-1.06)	0.06		
CRP (mg/L)	1.004 (1.00-1.007)	0.06		

Logistic regression for development of AKI grade 2-3 as opposed to AKI grade 0-1 in all patients. Variables recorded on admission. SAPS, sequential assessment physiology score; HBP, heparin-binding protein; WBC, white blood cell count; CRP, C-reactive protein; eGFRadm, estimated glomerular filtration rate on admission. Tyden J, Herwald H, Hultin M, Wallden J, Johansson J. Heparin-binding protein as a biomarker of acute kidney injury in critical illness. Acta Anaesthesiol Scand 2017; 61: 797-803. With permission from John Wiley and sons.

Paper III

Animal studies

HBP concentration in plasma did not differ significantly between the VILI group and the controls at any time of sampling. HBP concentration in BALF was significantly higher in the VILI group after 1 hour (p=0.04), 2 hours (p=0.03), 4 hours (p<0.01), and 6 hours (p=0.02) of ventilation (Fig.7).

The neutrophil count in BALF was higher in the VILI group at 2 hours (p<0.01) and 6 hours (p=0.03) of ventilation (Fig.8).

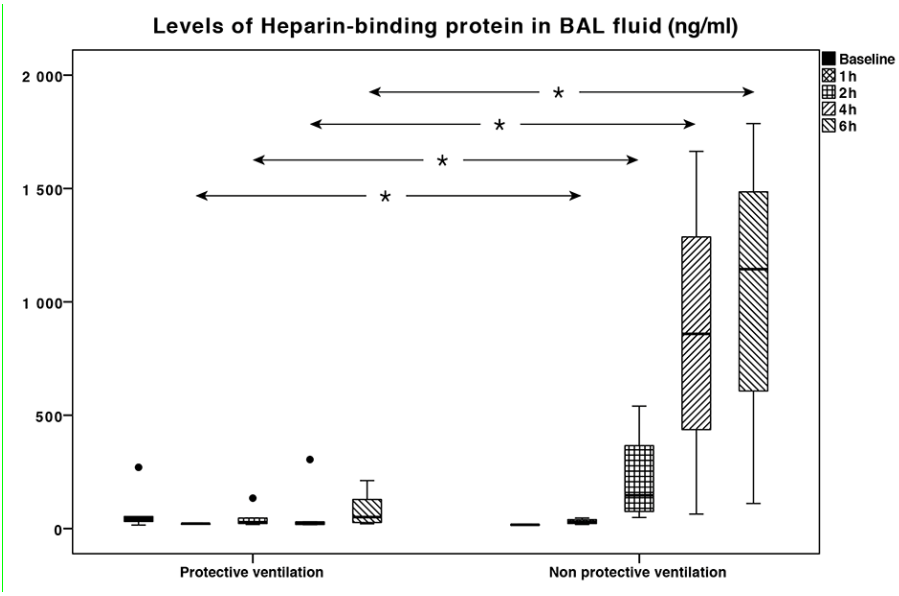

Figure 7. Concentration of heparin binding protein (HBP)(ng/ml) in bronchoalveolar lavage (BAL) fluid from pigs. HBP concentration was significantly higher in the group receiving harmful ventilation at 2 hours (p=0.03), 4 hours (p<0.01), and 6 hours (p=0.02) of ventilation. Boxes indicate the second to third quartile with the median. Brackets indicate min-max values, circles indicate outliers, and stars indicate differences for paired comparisons (p<0.05). Tyden J, Larsson N, Lehtipalo S, Herwald H, Hultin M, Wallden J, Behndig AF, Johansson J, (2018) Heparin-binding protein in ventilator-induced lung injury. Intensive Care Med Exp 6: 33.

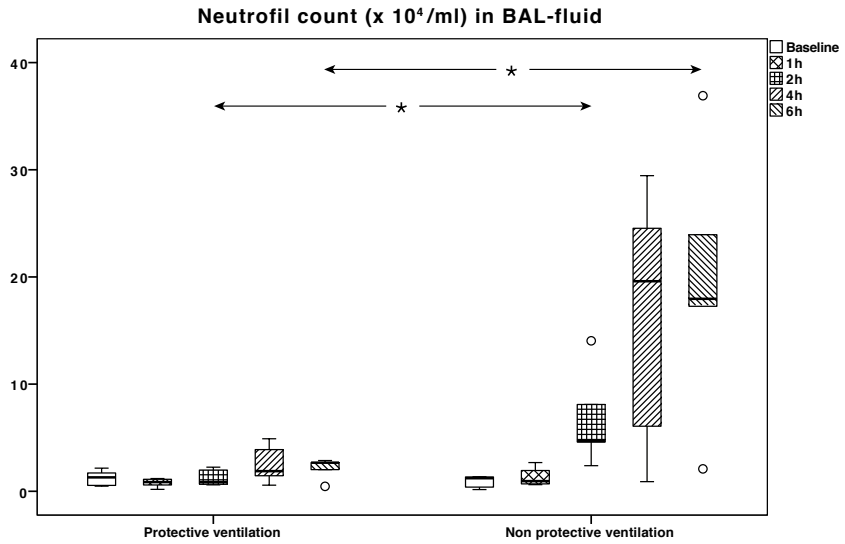

Figure 8. Neutrophil count (×10⁴/ml) in bronchoalveolar lavage (BAL) fluid. The neutrophil count was significantly higher in the group receiving harmful ventilation at 2 hours ($p<0.01$) and 6 hours ($p=0.03$) of ventilation. Boxes indicate the second to third quartile with the median. Brackets indicate the min-max values, circles indicate outliers, and stars indicate differences for paired comparisons ($p<0.05$). Tyden J, Larsson N, Lehtipalo S, Herwald H, Hultin M, Wallden J, Behndig AF, Johansson J, (2018) Heparin-binding protein in ventilator-induced lung injury. Intensive Care Med Exp 6: 33.

Human studies in healthy volunteers and ICU patients

The diagnoses and respiratory settings of the ICU patients are shown in Table 9.

The median HBP concentration in BALF from healthy volunteers was 0.90 ng/ml (IQR 0.79–1.01), and the median HBP concentration in BALF in intubated ICU patients was 1959 ng/ml (IQR 612–3306). The HBP concentrations were higher in the ICU patients ($p<0.01$).

Table 9. Characteristics and concentration of HBP in BALF and plasma in intubated ICU patients.

Diagnosis	SAPS3	FiO_2 (%)	Peak pressure (cm H_2O)	PEEP (cm H_2O)	Tidal volume (ml/kg)	HBP level in BALF (ng/ml)	HBP level in plasma (ng/ml)
Pneumococcal sepsis	76	50	26	9	6.3	25	1220
Aspiration pneumonia	65	35	15	8	6.3	1098	23
COPD+ pneumonia	91	40	22	9	5.6	1614	17
Staphylococcal pneumonia	83	55	28	11	6.1	1341	24
Fasciitis in extremity	61	45	26	12	6.3	4558	82
Status epilepticus	65	30	14	5	5.6	3254	29
Spinal trauma+ delirium	53	35	20	10	7.4	2325	47
Pneumonia	56	50	24	12	5.5	4646	
Aspiration pneumonia	51	60	17	8	6.8	2303	13
Pneumonia	68	35	15	7	5.9	251	34

SAPS 3: Simplified Acute Physiology Score 3. FiO2: fraction of oxygen in inspired gas. PEEP: positive end expiratory pressure. BALF: bronchoalveolar lavage fluid. HBP: heparin binding protein; COPD: Chronic obstructive pulmonary disease. Tyden J, Larsson N, Lehtipalo S, Herwald H, Hultin M, Wallden J, Behndig AF, Johansson J, (2018) Heparin-binding protein in ventilator-induced lung injury. Intensive Care Med Exp 6: 33.

Paper IV

Healthy volunteers

Eight healthy males aged 38 to 46 years were included. Median plasma concentration of HBP was 7.28 ng/ml (IQR 6.65-8.35 ng/ml). Median urine concentration was 0.87 ng/ml (IQR 0.68-0.94 ng/ml). Median urine flow was 1.64 ml/min (IQR 0.87-2.73 ml/min). The calculated median HBP clearance was 0.19 ml/min (IQR 0.08-0.33 ml/min).

Burn ICU patients

The median plasma concentration of HBP was 14.7 ng/ml (IQR 12.2-21.4). The median urine concentration of HBP was 2.44 ng/ml (IQR 1.59-

7.86). The median urine flow was 1.38 ml/min (IQR 0.96-2.43). Median HBP clearance was 0.30 ml/min (IQR 0.01-1.04).

In patients with Cystatin C > 1.44 mg/l median HBP clearance was 0.45 ml/min (IQR 0.15-2.81) whereas in patients with Cystatin C ≤ 1.44 mg/l median HBP clearance was 0.28 ml/min (0.14-0.55). Clearance of HBP was higher in the group with Cystatin C > 1.44 (p=0.04). There were no differences in clearance of HBP between healthy volunteers and burn patients with Cystatin C ≤ 1.44 mg/l (p=0.35).

CRRT patients

The median plasma concentration of HBP at start of CRRT was 155.4 ng/ml (IQR 45.3-220.6). At the time of changing the first drainage bag it was 91.3 ng/ml (IQR 47.3-287.0) and at the time of changing the second drainage bag it was 98.3 ng/ml (IQR 39.8-288.5) (Fig.9). There was no increase or decrease in plasma concentration of HBP between the first and last sampling time (p=0.14).

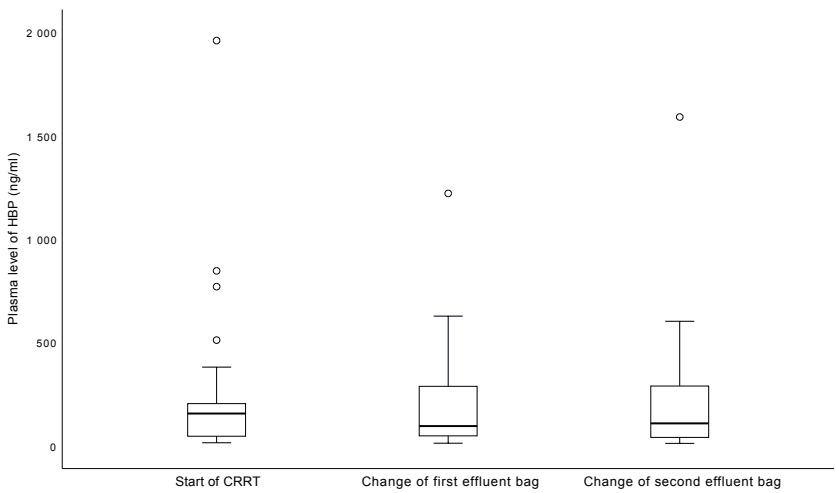

Figure 9. Plasma concentration of heparin binding protein (HBP) after the start of CRRT. Boxes indicate the second to third quartile with the median. Brackets indicate min-max values. Circles indicate outliers. There was no difference between first and last registration (p = 0.14).

The median concentration of HBP in the effluent flow was 9.1 ng/ml (IQR 7.8-14.4). The concentration of HBP in effluent flow related to plasma concentration is shown in figure 10. There was no correlation between the concentration of HBP in the effluent flow and plasma concentration of HBP (rho -0.14, p=0.45).

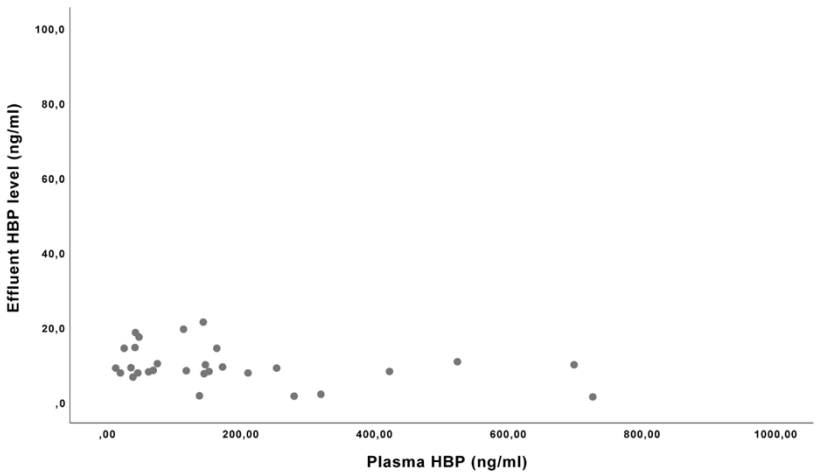

Figure 10. Concentration of heparin binding protein (HBP) in the effluent of CRRT in relation to plasma.

Discussion

HBP in relation to circulatory and respiratory failure

In Paper I, plasma concentration of HBP on admission to the ICU is associated with respiratory and circulatory failure but, for the individual patient, the predictive value of HBP is low. This is in an ICU study cohort with a large variety of diagnoses. The association between HBP concentration and circulatory and respiratory failure is consistent, with findings in subgroups of ICU patients in other studies[12,51,63,64,91].

While HBP concentration performs well in predicting organ failure in patients in the emergency department[49,50], these results can be interpreted as that this may not be so for patients on admission to intensive care.

There are at least a couple of possible explanations for this. Patients admitted to the ICU are typically more severely ill, with the immune system activated to a greater extent and most times with some degree of organ failure, when HBP concentration was measured. Consequently, the group not developing further organ failure in our study was probably more severely ill than the groups not developing organ failure in the studies on patients in the emergency deparment. This would reduce differences in HBP concentration between the groups developing/not developing further organ failure in our study.

It is also probable that for patients with suspected infection in the emergency department, the mechanisms for developing organ failure are more uniform than in ICU patients with mixed diagnoses where inflammatory response, while frequent, is not inherently the mechanism. This would result in patients with low HBP concentration and possible progression to severe organ failure.

In the subgroup with severe sepsis in Paper I, there was no association between respiratory failure and HBP concentration. This is in contrast to the study by Benzer et. al.[12] where such an association was observed. Our subgroup with sepsis was considerably smaller (83 vs 341 patients), and the patients in our group were also less severly ill, with an estimated mortality rate calculated from SAPS 3 of 45% versus an estimated mortality rate of 57% calculated from APACHE score. Based on the thoughts on severity of illness and more uniform mechanisms of development of organ failure in sepsis mentioned above, it is possible that the results in our subgroup might be a false negative.

Although increased vascular permeability and loss of plasma volume plays an important role in the development of circulatory failure associated with sepsis, the volume of fluid lost and the volume needed to be replaced has been debated lately[92]. Methods of evaluating circulatory failure in ours and several of the other studies on HBP concentration and circulatory failure are based on need for vasopressor therapy, which may reflect vasodilation rather than increased vascular permeability.

HBP and renal failure

In Paper II, plasma concentration of HBP sampled on admission to ICU was significantly higher in patients who developed AKI stage 3. The association between HBP concentration and the development of AKI stage 2-3 remained after adjustment in the regression model for age, sex, CRP, WBC, estimated GFR and disease severity (SAPS 3).

The ability of HBP concentration to predict the development of AKI was fair but not superior compared to using admission creatinine concentration.

The ability of predicting AKI in the subgroup with severe sepsis was high: ROC-AUC was 0.93 (95% CI 0.85-1.00) for HBP compared to 0.82 (95% CI 0.65-0.99) for admission creatinine. A major weakness is that there were only 59 patients with severe sepsis and only 9 of them developed AKI stage 2-3 during the first 7 days after admission. While this makes a generalization from this subgroup analysis highly speculative, these findings have been reproduced by two larger studies of septic AKI [75,76].

When studying AKI, early identification and identification of milder cases is difficult. The AKI definition used in this study is widely accepted, but a major part of it is based on the rise in creatinine. Elevation in creatinine does not appear until the glomerular filtration rate has dropped significantly. It is not known what impact a method for earlier identification of acute kidney injury could potentially have on these results, or what a comparison with any of the other used markers of kidney injury (other than creatinine) would reveal. Currently, creatinine level is part of the reference method for identification of kidney injury.

HBP and ventilatory induced lung injury

In Paper III with data from animals and humans, we show that in pigs subjected to VILI, concentration of HBP in BALF increased significantly over time, while HBP concentration in plasma did not in the first 6 hours. Human healthy volunteers had low HBP concentration in BALF, whereas HBP concentration was higher in intubated ICU patients who did not have documented VILI.

Although the presence of several inflammatory mediators in BALF in VILI have been reported previously [93], this is the first time that concentrations of HBP are described in this context. The known ability of HBP to increase vascular permeability[7] and development of lung injury in mice subjected to intravenous as well as endotracheal HBP[12,65] provides support for the idea that HBP could contribute to aggravating the lung injury associated with harmful ventilation.

In the ICU patients, HBP concentration in BALF was significantly higher compared to the healthy volunteers. This was despite seemingly non-injurious ventilation. This is probably due to inflammatory processes from a variety of causes other than VILI. High concentration of HBP has previously been found in BALF from lung transplant patients with respiratory infections[94], children with cystic fibrosis,[95] and calves with respiratory

syncytial virus infection[96]. It is unclear why HBP concentrations were high in the patients with status epilepticus and spinal trauma/delirium in our study. It is possible that regional overdistension of lung segments was present even though global tidal volumes appeared non-harmful.

In summary, while informative from a pathophysiologic perspective, measuring HBP concentration in BALF does not appear to be a promising way of reliably detecting injurious ventilation in ICU patients.

Excretion of HBP in urine and CRRT effluent

Healthy volunteers

In Paper IV, low concentrations of HBP in urine of healthy volunteers were observed. The concentrations were lower than those reported in a healthy control group of a previous study on urinary tract infections[78]. Calculated renal clearance of HBP was low compared to the normal creatinine clearance of 100 ml/min. Considering that HBP is a low-molecular weight protein sized 29 kDa, our results appear to be reliable. In general, low molecular weight proteins are freely filtered in glomeruli and almost completely reabsorbed by proximal tubular cells. With intact tubule cells, reabsorption will likely maintain a low concentration of HBP in urine.

Burn patients

In burn patients with normal concentrations of Cystatin C, our findings show that HBP clearance was not different to that of healthy volunteers. In patients with increased Cystatin C, the findings showed higher renal clearance of HBP. Urine concentrations of low molecular weight proteins depend on glomerular filtration and tubular reabsorption. Dysfunction in either or both entities of the kidney will alter the concentration, which is one possible explanation of the results.

Activated leukocytes in the urinary tract can release HBP and thereby increase the urinary concentration of HBP, as has been shown in patients with cystitis and pyelonephritis[78,79]. In our study of healthy volunteers, the urine was analyzed with urine dipsticks and microscopy to rule out these sources of HBP. However, in the burn ICU patients, presence of leukocytes in the urine was not analysed. This might be a possible source of error in our clearance calculations, possibly overestimating the clearance.

Even though HBP clearance was higher when renal function was impaired, clearance was still low. It seems unlikely that accumulation of HBP due to impared renal function needs to be a major consideration when evaluating HBP concentrations in plasma in previous or future studies.

CRRT patients

In ICU patients undergoing CRRT, we could demonstrate presence of HBP in the effluent fluid. The concentration of HBP appeared to be relatively constant despite large variations in plasma concentration of HBP.

Convective removal of a solute depends on transmembrane pressure, on molecular weight (MW) and structure of the solute, and also the cut-off point for filtration for the particular membrane. For the AN69 membrane, this averages 35 to 40 kD, which would permit passage for solutes like HBP (29 kD) and cytokines. De Vries et al. [97] investigated the relative contribution of membrane adsorption and convection on cytokine removal, and found that membrane adsorption represented the main clearance mechanism for cytokines. Other studies on inflammatory cytokines have shown elimination by convection as well as adsorption to the filter [98,99].

Our findings of relatively constant concentration in the effluent fluid despite variations of plasma concentration of HBP would support convection as an explanation rather than diffusion. However, we did not measure HBP adsorption to filters, which is a limitation in our study design.

We did not observe any consistent increase or decrease in plasma concentration of HBP when starting CRRT. On one hand, an increase in plasma concentration due to an inflammatory response to blood being exposed to the CRRT circuits would have been expected, since it is known that exposure to cardiopulmonary bypass increases HBP concentration[100]. On the other hand, a decrease due to removal by adsorption and convection may also be expected.

Given the association between high concentration of HBP in plasma and organ failure and mortality[12,51,63,75,101-104], a possibility to reduce HBP concentration could seem appealing. Our results suggest that starting CRRT with standard settings is not likely to be a successful way of doing this.

HBP as biomarker of sepsis in the critical care setting

In several studies, infections have been shown to trigger release of HBP in different contexts[9,79,80,105,106]. For patients presenting with a suspected infection in the emergency department, plasma concentration of HBP has been found to be a very good biomarker for the development of severe sepsis or septic shock[49,50]. More recently, similar results have been shown for unselected patients in the emergency department[107].

Using HBP concentration trying to identify patients with sepsis among patients admitted to intensive care has proven to be difficult. A possible explanation of this is a higher degree of activation of the immune system in non-septic ICU patients compared to non-septic patients in the emergency department. A summary of published studies where intensive care patients with severe sepsis or septic shock are compared to critically ill patients without severe sepsis or septic shock is shown below (Table 10).

Table 10. HBP as biomarker of sepsis in critically ill patients

Study	HBP concentration in sepsis, ng/ml	HBP concentration in non-sepsis, ng/ml	p	ROC AUC
Chew, Linder et al. 2012	27.2 (9.0–122.2)	24.1 (9.8–125.7)	0.71	na
Linder, Akesson et al. 2012	28 (3–1277)	14 (6-614)	0.029*	na
Dankiewicz, Linder et al. 2013	na	na	>0.05	na
Johansson, Brattstrom et al. 2013	8.0 (4.9–13.8)	7.9 (5.4–11.0)	0.67	0.55
Kaukonen, Linko et al. 2013	223 (54-332)	90 (64-134)	>0.05	na
Lin, Shen et al. 2013	18.62 (12.08-28.10)	16.51 (12.03-24.06)	0.68	na
Llewelyn, Berger et al. 2013	na	na	>0.05	0.58
Halldorsdottir, Eriksson et al. 2018	Day 1: 41.0 (23.4–58.6) Day 3: 40.0 (25.3–54.7) Day 5: 34.2 (27.0–41.5)	35.8 (26.4–45.3) 26.2 (22.5–29.9) 25.5 (20.9–30.1)	0.61 0.08 0.046*	0.6
Tyden, Herwald et al. 2016	47.7 (31.9-102.7)	34 (22.9-57.1)	<0.01*	0.64

HBP concentrations are presented as median with interquartile range or mean with 95% confidence interval as presented in the original articles. HBP, Heparin binding protein; ROC, receiver operating curve; AUC, area under the curve; na, not available. * indicates statistical significance $p<0.05$.

Future perspectives

On the basis on our findings, it appears that concentration of HBP alone is difficult to interpret in the critical care setting, probably because of pronounced activation of immunologic response both by the underlying disease itself and by interventions carried out in the ICU.

Studying HBP concentrations over time together with other markers of inflammation would be interesting as a way of following the progression of the inflammatory reaction, and possibly doing this over longer periods in the illness to try to find windows of opportunity for immunomodulation.

Attempts have been made to identify subgroups of ARDS based on biomarker profiles, identifying a "hyper-inflammatory" subgroup with different response to PEEP and fluid therapy, who have a higher predicted mortality[108,109]. Looking at HBP concentrations in this high morbidity and mortality subgroup would be a way of examining HBP involvement in this "hyper-inflammatory" subgroup.

Given the association of high HBP concentration and organ failure in several studies including ours, and the possible causal relationships demonstrated in 2 studies,[12,75] it is relevant to consider attempting to block HBP effects. However, this would have to be done with great caution, given the diversity of actions of HBP. An animal model of organ failure could give some clues to possible effects of blocking or eliminating HBP.

In a recent publication, heparin was found to counteract HBP's ability to increase vascular permeability in vitro[12]. This, together with several publications on heparin inhalation in acute lung injury with mixed results [110-112], point towards a possible way to attenuate lung injury caused by harmful ventilation and possibly lung injury from other causes. This could be a way of avoiding systemic blocking of HBP while theoretically achieving beneficial effects of the blockade locally in lungs.

Conclusions

- Plasma concentration of HBP in plasma on admission to the ICU is associated with respiratory and circulatory failure but the discriminating power to predict circulatory or respiratory failure in the individual patient is low.

- Plasma concentration of HBP on admission to ICU is associated with the development of severe acute kidney injury. The discriminating power to predict severe renal failure is low.

- In a model of ventilatory induced lung injury in pigs, the concentration of HBP in bronchoalveolar lavage fluid increases significantly over time compared to controls.

- When compared with healthy controls, HBP concentration in bronchoalveolar lavage fluid is high in intubated ICU patients in spite of non-harmful ventilation.

- In healthy individuals, calculated renal clearance of HBP is low and at the same level as in critically ill burn patients with normal kidney function.

- In critically ill burn patients with impaired kidney function, calculated renal clearance of HBP is increased.

- While HBP is found in the effluent fluid from CRRT, starting CRRT in critically ill patients does not consistently increase or decrease plasma concentration of HBP. Concentration of HBP in the effluent does not appear to be related to plasma concentration.

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