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BIOMARKERS OF ACUTE KIDNEY INJURY

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Stockholm 2011

Published by Karolinska Institutet.
Printed by Larserics Digital Print AB.

ISBN 978-91-7457-544-6

To Anja, Nils, Max, Astrid and Filip

What gets measured gets managed.

Peter Drucker

ABSTRACT

Acute kidney injury (AKI) is a common and potentially fatal complication in critically ill patients. The diagnosis relies on functional markers of decreased glomerular filtration rate (GFR) such as creatinine. Unfortunately, a rise in plasma creatinine lags behind the early structural changes that occur in response to various renal insults. Future treatment of AKI will most certainly be based on early biomarkers of structural damage. In addition, better real-time measures of GFR are needed to be able to monitor the course of the disease. Cystatin C outperforms creatinine as a marker of GFR in stable patients and human neutrophil lipocalin/neutrophil gelatinase-associated lipocalin (HNL/NGAL) has emerged as an early biomarker of AKI since it is readily synthesized by tubular cells following kidney damage. However, HNL/NGAL is also released by neutrophils in response to bacterial infections. Consequently, sepsis may affect HNL/NGAL concentrations in plasma and urine.

The aim of this thesis was to investigate the ability of HNL/NGAL and cystatin C to predict AKI and/or mortality in critically ill patients as well as to assess the impact of sepsis on HNL/NGAL and cystatin C levels in plasma and urine. In addition, we wanted to study the ability of two enzyme-linked immunosorbent assays (ELISAs) to detect HNL/NGAL released in urine from kidney epithelial cells and neutrophils, respectively, during the development of AKI.

Cystatin C predicted long-term mortality independently of AKI severity. Even in patients without AKI, elevated cystatin C was associated with increased mortality. During the first week in the intensive care unit cystatin C gradually increased, in patients both with and without AKI. This increase was similar in septic and non-septic patients. Cystatin C predicted sustained AKI, worsening AKI or death. HNL/NGAL in plasma was not predictive of AKI in patients with septic shock since sepsis *per se* increased plasma levels of HNL/NGAL. Urinary HNL/NGAL was less affected by sepsis and performed well as an AKI predictor. In combination, our two ELISAs effectively distinguished monomeric HNL/NGAL, released from kidney tubular cells, from dimeric HNL/NGAL, mainly released by activated neutrophils, during the development of AKI.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals as indicated below:

I. Bell M, Granath F, Mårtensson J, Löfberg E, Ekbom A, Martling CR.
Cystatin C predicts mortality in patients with and without acute kidney injury.
Nephrol Dial Transpl 2009;24(10):3096-102

II. Mårtensson J, Bell M, Oldner A, Xu S, Venge P, Martling CR.
Neutrophil gelatinase-associated lipocalin in adult septic patients with and without acute kidney injury.
Intensive Care Med 2010;36(8):1333-40

III. Mårtensson J, Martling CR, Oldner A, Bell M.
Impact of sepsis on levels of plasma cystatin C in AKI and non-AKI patients.
Nephrol Dial Transpl 2011 [e-pub ahead of print]

IV. Mårtensson J, Xu S, Bell M, Martling CR, Venge P.
Immunoassays distinguishing between human neutrophil lipocalin/neutrophil gelatinase-associated lipocalin released in urine from kidney epithelial cells and neutrophils.
Manuscript

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ABBREVIATIONS

ADQI	Acute Dialysis Quality Initiative
AKI	Acute kidney injury
AKIN	Acute Kidney Injury Network
APACHE	Acute Physiology And Chronic Health Evaluation
ARF	Acute renal failure
ATN	Acute tubular necrosis
AuROC	Area under the receiver operating characteristics curve
CKD	Chronic kidney disease
CRP	C-reactive protein
ELISA	Enzyme-linked immunosorbent assay
ESKD	End-stage kidney disease
GFR	Glomerular filtration rate
HNL	Human neutrophil lipocalin
ICU	Intensive care unit
IL	Interleukin
KIM	Kidney injury molecule
LMWP	Low-molecular-weight protein
MDRD	Modification of Diet in Renal Disease
MMP	Matrix metalloproteinase
NAG	N-acetyl-β-d-glucosaminidase
NFP	Net filtration pressure
NGAL	Neutrophil gelatinase-associated lipocalin
PCT	Procalcitonin
RIA	Radioimmuno assay
RIFLE	Risk, Injury, Failure, Loss of kidney function, End-stage kidney disease
ROC	Receiver operating characteristics
RRT	Renal replacement therapy
SIRS	Systemic inflammatory response syndrome
TBW	Total body water
TLR	Toll-like receptor

INTRODUCTION

An acute decline in the kidneys' ability to filter water and waste products commonly occurs in critically ill patients treated in the intensive care unit (ICU). It is reasonable to assume that this functional impairment is preceded by structural damage to the kidney epithelium, triggered by factors such as sepsis, major surgery and nephrotoxic drugs. This process, known as acute kidney injury (AKI) is strongly associated with increased mortality and survivors are predisposed to develop chronic kidney disease (CKD) sometimes progressing to lifelong dialysis dependency. While the prognosis of acute myocardial injury has improved over the years owing to the parallel identification of early biomarkers (e.g. troponins) of cardiac tissue damage, the recognition of AKI still relies on functional markers such as a rising plasma level of creatinine or a decline in urine output. These are late and unreliable measures and often do not indicate the injury before it is beyond repair.

A better understanding of the pathophysiology of AKI has emerged in recent years. Promising treatment strategies instituted before AKI is indicated by a rise in creatinine have been demonstrated in animal studies. In humans, however, efforts to treat AKI have been unsuccessful so far. An important reason for this therapeutic failure has been the lack of biomarkers, which detect the early stages in the AKI process. Several promising biomarkers have been identified, as representing different pathophysiological signals during the AKI continuum. Many of these biomarkers have shown excellent properties for the early detection of AKI. The performance has, however, been inconsistent in the studies. Before any biomarker can be introduced as a clinical tool in the ICU, it must be assessed in the presence of different critical conditions, e.g. in sepsis and after severe trauma.

This thesis focuses on human neutrophil lipocalin/neutrophil gelatinase-associated lipocalin (HNL/NGAL) and cystatin C as potential markers of AKI and kidney function, respectively. The impact of sepsis on the concentration of these biomarkers in plasma and urine is investigated as well as their ability to predict AKI and/or mortality in ICU patients. Finally, the ability of two enzyme-linked immunosorbent assays (ELISAs) to quantify the different molecular forms of HNL/NGAL released from kidney epithelial cells and neutrophils, respectively, is studied.

BACKGROUND

DEFINITION OF ACUTE KIDNEY INJURY

Acute renal failure (ARF) is a continuum of severity stages of kidney dysfunction ranging from a reversible decline in the glomerular filtration rate (GFR) to sustained ARF with anuria, which may progress to chronic renal failure. Plasma creatinine levels and changes in urine output have been used to define ARF for decades. The absence of a uniform definition has, however, impeded the ability to compare preventive strategies, therapies and outcomes in different studies.[1] In 2004 the Acute Dialysis Quality Initiative (ADQI) Group developed a new definition for ARF, called the Risk (R), Injury (I), Failure (F), Loss of kidney function (L) and End-stage kidney disease (E) (RIFLE) criteria.[2] Later, the term acute renal *failure* was replaced by acute kidney *injury* (AKI), reflecting the fact that structural injury most certainly precedes an acute decline in kidney function.[3]

The RIFLE criteria define AKI according to three stages of increasing severity (R, I and F) and two outcome criteria based on the duration of renal replacement therapy (L and E). The R, I and F classes are based on either a relative increase in plasma creatinine from baseline or an episode of oliguria (Table 1).

In 2007, the Acute Kidney Injury Network (AKIN) Group published a slightly modified version of the RIFLE criteria.[4] In the AKIN criteria, the outcome classes L and E were omitted and classes R, I and F were replaced by AKIN stages 1, 2 and 3. Based on findings that even small increments in creatinine are associated with adverse outcomes,[5, 6] an absolute increase of $\geq$ 26.4 µmol/l was included in stage 1. Patients starting renal replacement therapy (RRT) were included in stage 3, regardless of urine output or creatinine level.

Table 1. Definition and staging of AKI according to the Risk, Injury, Failure, Loss and End-stage kidney disease (RIFLE) criteria with modifications proposed by the Acute Kidney Injury Network (AKIN) Group.

AKI severity	Plasma creatinine criteria	Urinary output criteria
RIFLE		
Risk	≥ 1.5-fold increase in serum creatinine from baseline[†]	< 0.5 ml/kg/h for ≥ 6 h
Injury	≥ 2.0-fold increase in serum creatinine from baseline[†]	< 0.5 ml/kg/h for ≥ 12 h
Failure	≥ 3.0-fold increase in serum creatinine from baseline[†,‡]	< 0.3 ml/kg/h for ≥ 24 h or anuria ≥ 12 h
Loss of kidney function	Complete loss of kidney function > 4 weeks	
End-stage kidney disease	End-stage kidney disease > 3 months	
AKIN		
Stage 1	≥ 1.5-fold increase in serum creatinine from baseline[†] or an absolute rise in serum creatinine of ≥ 26.4 μmol/l within 48 h	< 0.5 ml/kg/h for ≥ 6 h
Stage 2	≥ 2.0-fold increase in serum creatinine from baseline[†]	< 0.5 ml/kg/h for ≥ 12 h
Stage 3	≥ 3.0-fold increase in serum creatinine from baseline[†,‡] or initiation of renal replacement therapy	< 0.3 ml/kg/h for ≥ 24 h or anuria ≥ 12 h

[†]When baseline creatinine is unknown it is recommended to estimate baseline using the simplified Modification of Diet in Renal Disease (MDRD) equation,[7] assuming a GFR of 75 ml/min/1.73 m^2.[2]
[‡]Patients with chronic kidney dysfunction reach class Failure or Stage 3 when creatinine increases ≥ 44 μmol/l from baseline to > 350 μmol/l.

INCIDENCE OF ACUTE KIDNEY INJURY

The incidence of AKI varies across studies depending on the definitions used and the populations studied. In 20,126 patients admitted to a tertiary hospital in Australia, almost 20% developed AKI defined by the RIFLE criteria.[8] In the ICU, the RIFLE criteria are fulfilled in 10–70% of patients, depending on the cohort under study.[9] After cardiac surgery the reported incidence is 19–45%.[10, 11] Approximately 4–5% of general ICU patients are treated with RRT in the ICU.[12] The population incidence of severe AKI treated with RRT has increased over the last few decades and reached around 250 per million population/year at the beginning of this decade.[13-16] This is roughly comparable with the population incidence of acute lung injury.[17] The overall population

incidence of AKI is ten-fold higher[13, 16] with a magnitude similar to that of severe sepsis.[18, 19]

OUTCOMES OF ACUTE KIDNEY INJURY

An increasing RIFLE stage is associated with increased length of ICU and hospital stay, higher mortality and higher health care costs.[9] Hospital mortality in a large ICU cohort was 8.8% in patients with RIFLE R, 11.4% in I and 26.3% in F. The corresponding mortality in non-AKI patients was 5.5%.[20] Uchino et al. found an increased in-hospital mortality risk (odds ratio), adjusted for a number of covariates, of 2.5, 5.4 and 10.1 in RIFLE R, I and F, respectively.[8] Even smaller absolute (26.4 µmol/l) or relative (25%) increases in creatinine are associated with adverse outcomes.[6] The reported mortality for patients treated with RRT in a Swedish general ICU was 46% after 30 days, reaching 60% after six months.[21]

Renal recovery is an important outcome measure in critically ill patients with AKI. Bell et al. showed that, among RRT-treated ICU survivors, 8–16% ended up in chronic dialysis.[22] Interestingly, in 2–4% of patients in whom RRT was successfully discontinued after ICU discharge, end-stage kidney disease (ESKD) later developed. The incidence of complete renal recovery (i.e. return to baseline GFR) in survivors has not been fully investigated. Information about the patients' baseline GFR is often missing and accurate GFR measurements (e.g. using iohexol-clearance) after ICU discharge are lacking in most studies. Schiffl et al. followed 226 survivors with RRT-treated AKI and found a complete recovery of GFR, estimated by creatinine, in 86% in patients surviving after 5 years.[23]

RISK FACTORS FOR ACUTE KIDNEY INJURY

The causal relationship between biological mechanisms and the structural and functional changes in AKI are difficult to investigate in humans, partly because kidney biopsies are rarely performed in ICU patients. Instead, we have to rely on associations between potential risk factors and AKI. Often multiple factors (patient-specific, treatment-specific or the effect of certain conditions commonly seen in the ICU, e.g. rhabdomyolysis, post-cardiac surgery and sepsis) act together to cause AKI in the ICU.

Patient-specific risk factors

Patient-specific risk factors include co-morbidities (e.g. diabetes mellitus, CKD, heart failure), advanced age and possibly certain gene polymorphisms.[24, 25] Especially, an increased risk of AKI in CKD patients is highlighted in several studies.[26] However, a similar or even higher in-hospital mortality in AKI patients without than in those with pre-existing CKD has been demonstrated.[12, 16, 27] This casts some doubt on CKD as a true risk factor for AKI. The observed associations between CKD and high AKI incidence might be confounded for several reasons.[28] First, valid measurements of baseline kidney function are absent in many studies. Second, other risk factors for AKI such as diabetes and heart failure are common in CKD. Finally, inclusion of RRT requirement in the AKI definition may produce a selection bias since CKD patients are likely to have high serum creatinine, a common RRT indication.

Treatment-specific risk factors

Nephrotoxic drugs contribute to almost 20% of severe AKI cases in ICU patients.[12] Some drugs like aminoglycosides and contrast dye are innately nephrotoxic and exert their effect by impairing mitochondrial function, increasing oxidative stress or forming free radicals. Contrast-induced nephropathy is reported as one of the most prevalent causes of AKI in hospitalized patients.[29] Drugs such as vancomycin may induce an immune response in the kidney interstitium leading to interstitial nephritis.

Rhabdomyolysis

Bywaters and Beall discovered the association between crush injury and renal impairment in 1941.[30] Rhabdomyolysis is characterized by the breakdown of skeletal muscle with the release of muscle-cell contents, including myoglobin, into the bloodstream. The reported incidence of AKI in rhabdomyolysis is substantial (13–50%).[31] Most patients with rhabdomyolysis-induced AKI do, however, recover their kidney function.[32] Several mechanisms interact in rhabdomyolysis-induced AKI. Myoglobin is a 17-kDa oxygen-carrying heme protein that contains ferrous (Fe^{2+}) oxide. Myoglobin is completely reabsorbed and metabolized by the proximal tubule after free glomerular filtration but appears in urine if the reabsorptive capacity is exceeded. Oxidation of ferrous to ferric (Fe^{3+}) oxide promotes the generation of free oxygen radicals that cause proximal tubular damage. Myoglobin can also precipitate

and form heme-pigment casts within the tubular lumen, eventually leading to tubular obstruction. These processes are enhanced by intravascular volume depletion and an acidic tubular milieu. Circulation of inflammatory mediators and activation of the immune system may also play a role.[31]

Cardiac surgery

Cardiac surgery is the second most common trigger of AKI.[12] Several mechanisms behind this association have been suggested with a focus on the impact of the cardiopulmonary by-pass (CPB) circuit.[33] Pump-induced haemolysis with the release of free iron has been suggested. Free iron is toxic to the tubular epithelium and may impair cell proliferation and hence the repair process in AKI.[34] The formation of free oxygen radicals catalysed by free iron addressed in the previous section may also be a feature of CPB-induced AKI.

Sepsis and the systemic inflammatory response

The word sepsis is used to describe the syndrome of systemic inflammation when infection is the cause.[35] Severe sepsis refers to conditions when sepsis is complicated by organ failure and septic shock is characterized by hypotension resistant to volume resuscitation. An annual increase of sepsis cases close to 9% has been reported from the United States over the last decades.[19] ICU mortality in patients with severe sepsis and septic shock was above 30% and 50%, respectively, in a multi-centre study from Europe.[36] The inflammatory response is important to survive a severe infection but may also cause organ damage. The innate immune system is the first line of defence against invading microbial pathogens. Toll-like receptors (TLRs) are located on the cell surface of many human cell types and recognize unique structures on the cell wall of microorganisms. Binding to TLRs rapidly induces the production and release of pro-inflammatory (e.g. TNF-α and IL-1β) and anti-inflammatory (e.g. IL-10) cytokines and chemokines. Alternatively, activation of TLRs directs the cell towards apoptosis. Pro-inflammatory cytokines up-regulate adhesion molecules on endothelial cells and, together with chemokines, facilitate recruitment and adhesion of neutrophils to the endothelium. Subsequent release of prostaglandins, leukotrienes, proteases and oxidants from activated immune cells impairs the key functions of the endothelium, namely its selective permeability, vasoregulation and provision of an anticoagulant

surface. Hence, widespread endothelial injury results in vasodilation, increased vascular permeability and coagulopathy with the risk for microthrombi formation. Vasodilation is further enhanced by increased synthesis of nitric oxide (NO).[37] Severe sepsis is the most common AKI trigger in ICU patients, contributing to approximately 50% of cases. Besides, septic AKI is associated with higher mortality and prolonged length of stay as compared to non-septic AKI.[12, 38]

PATHOPHYSIOLOGY OF ACUTE KIDNEY INJURY

Tubular injury

The pathophysiology of human AKI is not fully understood. Instead, we rely on animal models to solve pieces of the AKI puzzle. The traditional view of AKI, and its cause, has been focused on renal ischaemia, triggered by haemodynamic instability and subsequent renal vasoconstriction, resulting in acute tubular necrosis (ATN). Lately, the concept of tubular ischaemia as the sole explanation for AKI, especially in sepsis, has been challenged.[39]

Regardless of the type of insults (ischaemia, endotoxins, nephrotoxic agents) behind different forms of AKI, inflammation seems to play a key role in the pathophysiology.[40] The kidney insult initiates a pro-inflammatory response in the proximal tubular and endothelial cells, resulting in the release of cytokines and chemokines. Vasodilation, increased vascular permeability and up-regulation of adhesion molecules (e.g. P-selectin) in peritubular capillaries facilitate recruitment and subsequent migration of inflammatory cells (neutrophils, lymphocytes and macrophages) into the kidney interstitium. During transmigration, the neutrophils release their granular contents, including cytokines, causing further damage to the kidney epithelium.[41-44]

Injured tubular cells detach from the underlying basement membrane when cytoskeletal integrity and cell polarity is lost.[45] If the injury is severe enough, viable as well as apoptotic and necrotic cells are desquamated into the tubular lumen, leaving parts of the basement membrane denuded. This could potentially allow back leak of fluid, causing interstitial oedema.[46]

In response to injury, adjacent viable tubular cells initiate an immediate repair process. Repair either successfully restores the functional integrity of the nephron or result in fibrotic lesions and chronic kidney dysfunction. Normal repair includes migration and division of surviving cells to replace lost cells, so-called de-differentiation (Figure 1). Finally, these cells re-differentiate and regain cytoskeletal integrity and polarity.[47] Delivery of iron to tubular cells appears to be involved in this process.[48]

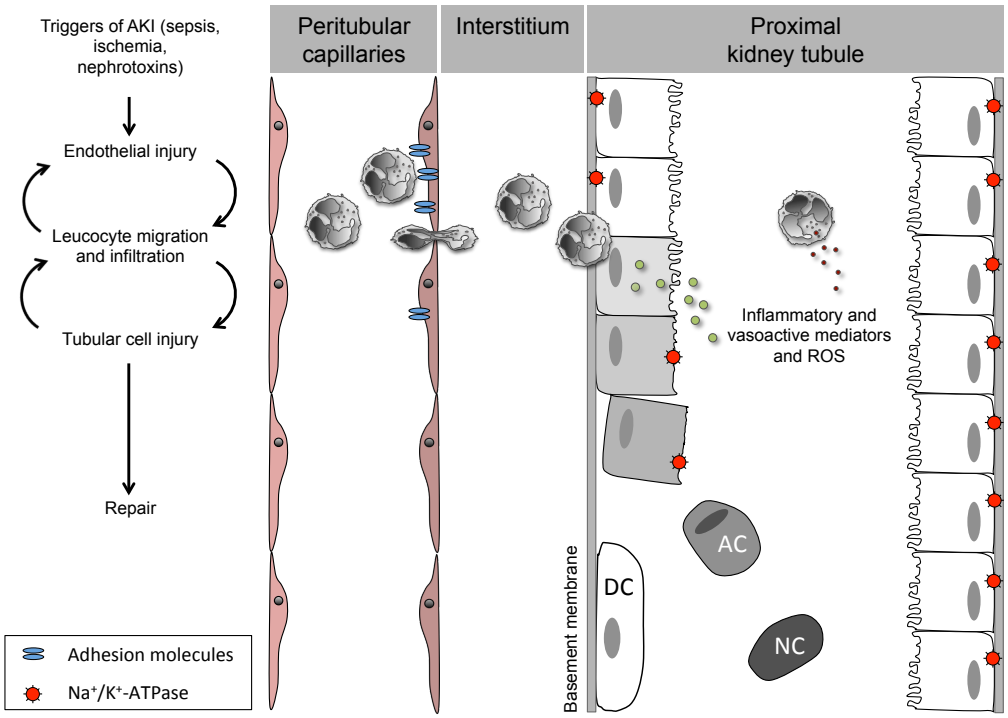

Figure 1. Pathophysiological mechanisms of acute kidney injury and repair. Adhesion molecules up-regulate on the surface of endothelial cells and facilitate migration of neutrophils into the kidney interstitium and tubular lumen. Inflammatory and vasoactive mediators and reactive oxygen species (ROS) damage the tubular cells. Shedding of the proximal tubule brush border, loss of polarity with mislocation of Na^+/K^+-ATPase as well as apoptosis and necrosis may occur. With severe injury, viable and non-viable cells are desquamated, leaving parts of the basement membrane denuded. Inflammatory and vasoactive substances released from the injured tubular cells worsen the pathophysiological changes. If the repair process is successful, viable cells de-differentiate and spread to cover exposed areas of the basement membrane and restore the functional integrity of the nephron. AC, apoptotic cell; DC, de-differentiating cell; NC, necrotic cell.

Impaired glomerular filtration

Filtration of water and solutes in the kidneys depends on the net filtration pressure (NFP) over the glomerular filtration barrier and the unique properties of the filtration barrier itself. NFP is the driving force for filtration which is mainly determined by the systemic blood pressure and by the relative tone in the afferent and efferent arterioles and is opposed by the oncotic pressure (determined mainly by the albumin concentration) in plasma and the hydrostatic pressure in Bowman's space. The magnitude of the normal renal blood flow (~25% of cardiac output) is in excess of the kidney's metabolic needs in order to maintain a high GFR. Despite fluctuations in systemic arterial pressure that would be expected to influence NFP, GFR changes very little in the healthy kidneys. This is a consequence of intrarenal *autoregulation,* whereby the resistance in the afferent arterioles, and hence the NFP, changes as the systemic blood pressure changes. Two mechanisms underlying autoregulation have been proposed: (1) the afferent arteriolar wall contracts in response to being stretched when the blood pressure increases, the so-called *myogenic reflex*; (2) GFR initially increase in response to increased blood pressure, leading to an increased salt delivery to the 'salt-detecting' macula densa cells in the distal part of the nephron. The increased need for tubular reabsorption of salt is energy consuming and it is followed by the release of signalling substances (e.g. adenosine) which contract the afferent arteriole thereby blunting the increase in renal blood flow and GFR, so-called *tubulo-glomerular feedback*. These autoregulatory mechanisms hence allow GFR to be maintained across a wide range of mean arterial pressures (80–180 mmHg). Outside the autoregulatory range, e.g. in circulatory shock, renal blood flow and GFR change with blood pressure.

To enter Bowman's space, water and solutes must pass through the fenestrated endothelium, basement membrane and the slit-pores between podocytes (specialized cells lining the glomerular capillaries; Figure 2).

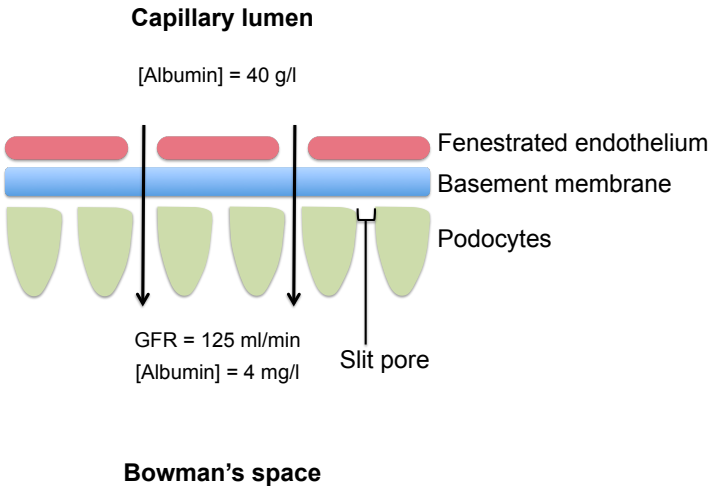

Figure 2. Schematic illustration of the glomerular barrier. The barrier is negatively charged and obstructs the passage of negatively charged proteins such as albumin. In the healthy kidney only small amounts of albumin pass through the glomerular filter. The albumin concentration [Albumin] in plasma and glomerular filtrate is shown.

The fenestrated endothelium is size-selective and could theoretically allow passage of molecules up to a diameter of 8 nm (80 Å). Yet, albumin has a diameter of 6 nm and still only < 0.1% is filtered. This is because the filtration barrier is negatively charged. Since many plasma proteins are negatively charged they will be more or less repelled by the filter membrane.[49] Mesangial cells are also found in the renal glomerulus and have features similar to smooth muscle cells. These cells are targets for vasoactive substances and are able to regulate the filtration area and hence GFR.[50]

What impairs GFR and when it occurs during the AKI process is not fully understood. Decreasing GFR is a physiological response to haemodynamic instability/low blood pressure and can occur without injury to the kidney epithelium. A low GFR is also the major functional event of AKI. Despite this fact, current research has focused on tubular rather than glomerular injury when attempting to explain decreased GFR in AKI. Renal histopathology has been investigated in AKI patients and, surprisingly, despite a complete loss of kidney function in some of these patients, only moderate structural changes have been observed.[51-53] The mechanisms behind the AKI related decrease in GFR might well be other than structural changes. Recently, alterations in renal haemodynamics and functional changes in the tubules, have been proposed to be important factors.

Mislocation of tubular Na^+/K^+-ATPase, resulting in impaired sodium reabsorption, has been observed in AKI.[45] Consequently, increased sodium delivery to the macula densa will enhance the tubulo-glomerular feedback, reducing GFR and salt delivery to dysfunctional tubular cells. Interestingly, in a septic AKI model, global renal blood flow was markedly increased despite a significant reduction of GFR.[54] A more pronounced vasodilation in efferent as compared to afferent arterioles, resulting in a lower NFP, may explain this. The injured kidney endothelium responds more vigorously to endogenous vasopressors and less to vasodilators. Together with the formation of microthrombi and tissue oedema, microvascular congestion may follow. Finally, tubular obstruction by desquamated cells as well as dysfunction of the charge- and size-selectivity of the glomerular filtration barrier may be involved in AKI.[46, 55]

BIOMARKERS OF ACUTE KIDNEY INJURY

In general, the purpose of a biological marker (biomarker) is to measure pathological processes or pharmacological responses to therapeutic interventions.[56] So far, efforts to treat AKI have failed in humans.[57] A very important reason is the lack of markers to identify the early pathological processes at a time-point when treatment might be successful. This section compares the strengths and weaknesses of creatinine and cystatin C in plasma as *functional* markers of GFR. Furthermore, some promising novel biomarkers of acute kidney *injury* are summarized. Their potential roles in the pathophysiology of AKI will be outlined.

Creatinine

In 1886 Max Jaffé observed the red colour formed when creatinine reacted with picric acid.[58] The Jaffé reaction has withstood the test of time and is still in use. The use of creatinine as a marker of GFR was investigated for the first time by Poul Brandt Rehberg, who in 1926 studied renal clearance of orally administered creatinine.[59] Determination of endogenous creatinine clearance was, however, precluded until 1938.[60] Creatinine is a 113-Da amino acid compound derived from the conversion of creatine in skeletal muscle. Creatine is mainly synthesized in the liver, but is also supplied from our diet (mainly meat). Muscle contains 98% of the total creatine pool in the body. The conversion to creatinine is a relatively stable process that is proportional to the total muscle-cell mass. Creatinine is distributed throughout the total body water

(TBW), it is not protein-bound and it is mainly excreted via glomerular filtration. There are several limitations to the use of plasma creatinine as a marker of GFR, especially in ICU patients. First, a rapid loss of muscle mass is common in critically ill patients probably as an effect of immobilization[61] and/or catabolism.[62] A decreasing creatine pool, resulting in lowered plasma creatinine levels over time, could, theoretically, be a consequence of this. Second, increased tubular secretion compensates a fall in GFR and plasma creatinine may not rise until GFR is halved. Extrarenal clearance, via intestinal elimination, may also be substantial when GFR is reduced.[63] Third, owing to its large distribution volume (TBW), it takes time before steady state is reached after rapid changes in kidney function or hydration status. Forth, creatinine production and release into plasma depends on many non-renal factors (e.g. muscle mass, liver function and dietary intake) and results in individual variations in baseline creatinine. Fifth, pathological conditions can increase (e.g. rhabdomyolysis) or decrease (e.g. liver failure) plasma levels independently of kidney function.[63] Finally, the drawbacks of the Jaffé reaction must also be acknowledged. Pseudocreatinines (e.g. ketone bodies, glucose, cephalosporins) may significantly affect the colorimetric reaction and give falsely elevated creatinine readings. This might have an impact when kidney function is monitored in critically ill patients.

GFR measuring from endogenous creatinine clearance does not offer an advantage over serum creatinine alone. GFR can be estimated by calculating endogenous creatinine clearance (Cl_{Cr}) from:

$$Cl_{Cr}\ (ml/min) = U_{Cr} \times V/P_{Cr},$$

where U_{Cr} and P_{Cr} are the creatinine concentration in urine and plasma, respectively, and V is the urinary flow rate. When GFR falls, P_{Cr} will initially be unchanged and U_{Cr} will increase due to increased tubular secretion, hence Cl_{Cr} will overestimate GFR. Errors in the measurement of urine volume will also limit the accuracy.

Cystatin C

Cystatin C is a 13-kDa molecule considered to be produced by all nucleated cells at a constant rate, unaffected by such factors as muscle mass and diet. Being a potent inhibitor of cysteine proteases, it prevents breakdown of extracellular proteins. After free filtration, cystatin C is reabsorbed and subsequently catabolized by the proximal tubular cells. Hence, cystatin C has many potential features of an ideal marker of GFR. Indeed, several studies have shown that cystatin C outperforms creatinine as a marker of GFR in stable patients.[64-66]

A number of non-renal factors affecting cystatin C levels in plasma have recently been identified, however. Glucocorticoid treatment increases cystatin C levels in a dose-dependent manner.[67-69] Thyroid dysfunction must also be taken into account when interpreting cystatin C results since the levels increase in hyperthyroid and decrease in hypothyroid patients.[70] Restoration of thyroid function seems to normalize cystatin C concentrations.[71]

Cystatin C is more than 100 times larger than creatinine. Small reductions in the glomerular pore size might impair the passage of cystatin C, whereas smaller molecules, like creatinine, pass freely. Therefore, cystatin C could theoretically be more sensitive to mild changes in GFR than creatinine. Contradictory to this are the recent findings that sepsis and ischaemia induce changes in the glomerular filtration barrier which increase clearance of large molecules.[55, 72]

The performance of cystatin C in plasma in predicting AKI has been investigated in various settings and the results from general ICUs have varied widely. In a study by Herget-Rosenthal et al., the rise in cystatin C preceded creatinine in ICU patients who were developing AKI. In fact, a > 50% rise predicted AKI within 24 h with an area under the receiver operating characteristics curve (AuROC) of 0.97.[73] Moreover, Nejat et al. observed that cystatin C increased before creatinine more often than vice versa in AKI patients and predicted sustained AKI with an AuROC of 0.80.[74] The ability to predict mortality or subsequent need for RRT was, however, moderate (AuROC, 0.61) and no better than for creatinine. Royakkers et al. found serum cystatin C to be a poor predictor of AKI (AuROC, 0.62) and the need for RRT (AuROC, 0.66).[75] Likewise,

Perianayagam et al. showed that cystatin C, creatinine and urea in serum as well as urine output were equally poor predictors of dialysis requirement or in-hospital mortality at the time of nephrology consultations with AKI patients (AuROC, 0.602–0.665).[76] The predictive accuracy of plasma cystatin C was recently reviewed in a meta-analysis including 13 studies from a wide range of settings and age groups. The overall accuracy was impressively high with an AuROC of 0.96.[77]

It is controversial as to whether inflammation has an impact on plasma cystatin C levels. Lysosomal cysteine proteases are released in response to trauma and sepsis and are involved in apoptosis.[78, 79] It is possible that the role of cystatin C is to protect cells from increased protease activity and cystatin C could, at least in theory, be either up-regulated or decreased due to consumption. Two large cross-sectional studies found a significant association between cystatin C and systemic inflammation measured by CRP.[80, 81] On the other hand, Grubb et al. found no temporal changes in cystatin C in patients with systemic inflammation induced by surgery.[82]

Filtered cystatin C is, like many low-molecular-weight proteins (LMWPs), endocytozed via the megalin receptor located at the apical membrane of proximal tubular cells.[83] Impaired reabsorption is a feature of AKI and leads to an accumulation of cystatin C in the urine. Down-regulation of the megalin receptor probably contributes to this. The effect of albuminuria *per se* on urinary cystatin C levels has recently been discussed. Competitive inhibition of the megalin receptor by albumin may decrease the tubular uptake of cystatin C (and other LMWPs).[84, 85] Sepsis, even without AKI, may be associated with albuminuria and could therefore cause elevated cystatin C levels in urine. Nejat et al., who found higher urinary cystatin C levels in septic as compared to non-septic patients, supported this theory.[86]

Urinary cystatin C predicted RRT requirement in patients classified as having non-oliguric ATN with an AuROC of 0.92.[87] The performance in predicting AKI was investigated in a recent meta-analysis. The pooled AuROC from four studies amounted to 0.64.[77]

Human neutrophil lipocalin/Neutrophil gelatinase-associated lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL), also known as human neutrophil lipocalin (HNL) or lipocalin 2, was first purified from the secondary granules of human neutrophils.[88, 89] The protein was identified as a 25-kDa monomer, as a 45-kDa disulphide-linked homodimer and as a 135-kDa heterodimer, covalently conjugated with gelatinase (matrix metalloproteinase (MMP)-9).[88, 89] HNL/NGAL is synthesized in the bone marrow during myelopoiesis and is directed to and stored in the neutrophil granules.[90] Mature neutrophils release HNL/NGAL into the bloodstream in response to bacterial infections. In fact, significantly higher HNL/NGAL levels in serum and plasma are seen in bacterial, as compared to viral, infections.[91, 92] HNL/NGAL mRNA is also expressed in other human tissues frequently exposed to microorganisms, such as colon, trachea, lung and kidney tissues.[93] Stimulation with the inflammatory mediator IL-1β increased HNL/NGAL synthesis in a number of human cell lines.[94] Additionally, elevated plasma levels have been observed in several inflammatory conditions such as acute peritonitis and acute exacerbations of obstructive pulmonary diseases.[95]

In the search for novel biomarkers of AKI, HNL/NGAL was among the most up-regulated genes after ischaemic AKI in animal models.[96, 97] The subsequent proteomic analyses verified that HNL/NGAL was highly induced in animal kidneys following ischaemic and nephrotoxic AKI and that urinary concentrations increased several-fold early on (within hours) after the insult.[97, 98] An accumulation of HNL/NGAL in serum and urine in humans with established AKI was later revealed.[99]

The first study evaluating HNL/NGAL as an AKI *predictor* was conducted on children after cardiac surgery. Urinary HNL/NGAL rose almost 100-fold and serum HNL/NGAL 20-fold up to 48 h before AKI was detected by creatinine. The urinary HNL/NGAL level was an almost perfect AKI predictor with an AuROC of 0.998.[100] Since obtaining these encouraging results, the predictive performance of HNL/NGAL has been tested in various clinical settings. The results have varied across studies and can be ascribed to a number of factors. First, the definition of the outcome variable, i.e. AKI, is important. This was shown in one study where the predictive value increased with the severity of AKI ranging from an AuROC of 0.65 for the prediction of a > 25% increase in creatinine to an AuROC of 0.79 for predicting a rise above 50%.[101] Second,

inclusion of CKD patients may affect the results since CKD *per se* is associated with elevated serum and urinary HNL/NGAL levels.[102, 103] McIlroy et al. found that urinary HNL/NGAL only identified AKI after cardiac surgery in patients with an estimated baseline GFR of > 90 ml/min.[104] Third, the time from HNL/NGAL measurement to AKI development differs among studies. It is reasonable to believe that the predictive value will be higher if AKI develops within 1 day, rather than within 10 days, from the measurement. Forth, co-morbid diseases and conditions such as sepsis might affect HNL/NGAL levels independently. In fact, Bagshaw et al. found higher HNL/NGAL in plasma and urine in AKI patients with sepsis as compared to non-septic AKI patients despite equal AKI severity.[105]

The predictive performance was recently highlighted in a meta-analysis pooling data from 19 studies and eight countries involving 2,538 patients. The overall AuROC for AKI prediction was 0.815 and was similar in general ICU patients and after cardiac surgery. Moreover, HNL/NGAL measured in urine or plasma/serum performed equally well using the proposed cut-off value of 150 μg/l. The predictive ability was better in children (AuROC, 0.930) than in adults (AuROC, 0.782), probably reflecting the impact of co-morbid illness on HNL/NGAL levels.[106]

Normally, small amounts of HNL/NGAL are produced by different tissues and released into the bloodstream. After free filtration, HNL/NGAL is reabsorbed via megalin-receptor mediated endocytosis by the proximal tubule.[99, 107] Elevated HNL/NGAL levels in urine and plasma during early AKI may have several causes (Figure 3): (1) impaired reabsorption in the damaged proximal tubule increases urinary levels;[99] (2) induced synthesis in different parts of the nephron has been demonstrated in animal models;[99, 108] (3) secretion from neutrophils, migrating from capillaries into the tubular lumen, may also be a potential source;[41] (4) increased HNL/NGAL mRNA expression has been found in distant organs (lung) in animal AKI models.[109] Such extra-renal production may contribute to elevated plasma levels in AKI. A subsequent decline in GFR will further amplify plasma levels. Worth noting is that HNL/NGAL is also highly expressed and released by the liver and circulating neutrophils in response to inflammation.[110, 111] This might increase levels of plasma and urinary HNL/NGAL irrespective of any potential kidney damage. Moreover, increased urine levels have

been observed in patients with urinary tract infection (UTI), although at lower levels than normally seen in AKI.[112]

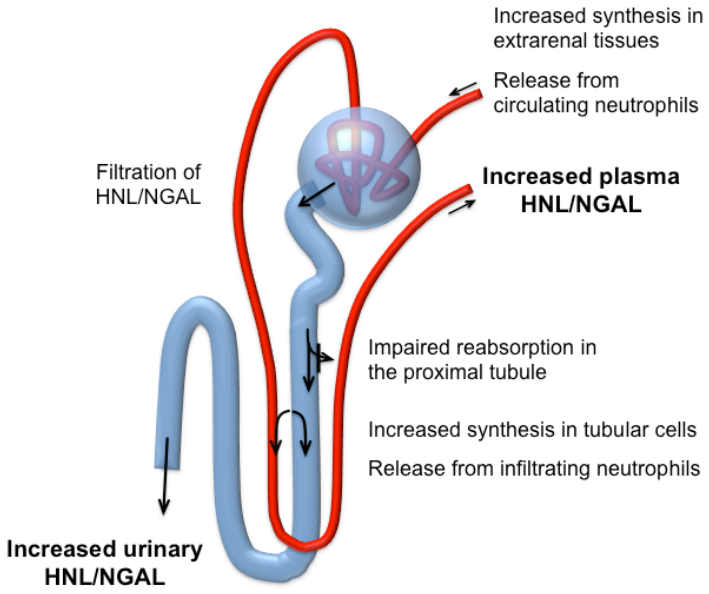

Figure 3. Proposed mechanisms for increased HNL/NGAL in plasma and urine in AKI.

The results from a recent in vitro study on human tubule epithelial cells (HK-2 cells) and neutrophils indicate that kidney epithelial cells mainly secrete monomeric HNL/NGAL, whereas neutrophils mainly release the dimeric form detected by Western blot.[113] The finding that dimeric HNL/NGAL was the predominant form in the urine of patients with UTI further supported this possibility. The different forms of HNL/NGAL (monomeric, dimeric, heterodimeric) may expose different epitopes. The choice and configuration of antibodies directed against different epitopes on the HNL/NGAL molecule clearly have an impact on the clinical performance of the assay.[114] Notably, most studies investigating HNL/NGAL as an AKI predictor use commercial ELISAs such as Bioporto (Gentofte, Denmark) and R&D systems (MN, USA). These assays are based on monoclonal anti-HNL/NGAL antibodies. The use of different assays may contribute to the varying performance of NGAL as an AKI predictor in the studies.[106] Whether HNL/NGAL in urine during AKI is derived from both tubular cells and neutrophils, and whether a possible contribution from these sources represent different pathophysiological signals during AKI development, has not yet been addressed.

Several biological functions for HNL/NGAL have been suggested. By its ability to bind siderophores (small iron-binding molecules), HNL/NGAL is involved in the iron transport to and from cells.[115] Iron is vital for cell survival but may also be toxic owing to its ability to catalyse the conversion of hydrogen peroxide to free oxygen radicals. Bacteria release siderophores in order to acquire iron from the host. HNL/NGAL acts as a bacteriostatic agent by sequestering iron, which is vital for bacterial growth.[110, 116] Neutrophils, which store HNL/NGAL in their granules, provide the organism with a mobile source of HNL/NGAL. Furthermore, HNL/NGAL production in epithelial cells may be important for the local defence against an infection. Iron is also necessary for the proliferation and differentiation of human cells. Yang et al. found that HNL/NGAL promoted iron-dependent differentiation of mesenchymal progenitors into complete nephrons during the development of the kidneys.[48] The renoprotective role of HNL/NGAL is supported by animal models of ischaemia-reperfusion-induced AKI, where intravenously administered HNL/NGAL was rapidly taken up by proximal tubular cells and reduced tubular damage and apoptosis as well as increased cell proliferation.[99, 117]

α_1-microglobulin

α_1-microglobulin is a 27-kDa glycoprotein produced by the liver.[118] It exists in plasma in a free monomeric form but also bound to other proteins, mainly IgA. α_1-microglobulin is found in the connective tissue of most organs. Its exact biological role is still unknown, but anti-inflammatory properties have been suggested. The free form is eliminated through glomerular filtration and is then reabsorbed and catabolized by the proximal tubular cells. Hence, increased plasma levels are related to impaired kidney function, whereas decreased levels are seen in liver failure.[118] Free α_1-microglobulin is detected in urine and is a marker of proximal tubular dysfunction.[119] Albuminuria, e.g. in response to systemic inflammation, may increase the urinary excretion of α_1-microglobulin by competition on the megalin receptor.[120, 121] This might increase the threshold for detection of AKI.

In one study on non-oliguric patients with established AKI, α_1-microglobulin predicted the subsequent need for RRT with an AuROC of 0.86.[87]

Kidney injury molecule-1

Phagocytosis of apoptotic and necrotic cells in the tubular lumen might down-regulate the pro-inflammatory response and aid in the repair process during AKI. Kidney injury molecule (KIM)-1 is a transmembrane glycoprotein that is up-regulated on the tubular epithelial cell surface in response to injury. KIM-1 seems to transform these cells into phagocytes which clear the lumen of cellular debris.[122] Additionally, KIM-1 is expressed in tubular cells undergoing de-differentiation, further supporting its role in the repair process after injury.[123] Its ectodmain is cleaved and released in urine and can be measured as a biomarker of AKI.[124] In a recent study KIM-1 outperformed creatinine as a predictor of drug-induced tubular damage classified according to histopathological changes in rats.[125] In a study on six patients with biopsy-proven ATN, KIM-1 was highly expressed in the proximal tubules.[124] The same study also found that urinary KIM-1 was significantly higher in patients with 'ischaemic' ATN as compared to those with other acute and chronic kidney diseases.

Interleukin-18

Interleukin (IL)-18 is a pro-inflammatory 18-kDa cytokine produced and secreted by proximal tubular cells and leucocytes in AKI.[40] Pro-IL-18 is converted to its active form by a cysteine protease (caspase-1). The finding that experimental inhibition of IL-18 in animals protected against ischaemic renal injury supported its role in the pathogenesis of AKI.[126] Mature IL-18 does not seem to interfere with the megalin-receptor.[85]

N-acetyl-β-d-glucosaminidase

N-acetyl-β-d-glucosaminidase (NAG) is a large (> 130 kDa) lysosomal enzyme found in several human cells, including the renal tubules. NAG is the most active glycosidase found in proximal tubular epithelial cell lysosomes. It is not filtered over the glomeruli and elevated levels in urine are therefore believed to reflect tubular injury. Since elevated urinary NAG levels have been shown during several active renal diseases, the specificity for AKI might be reduced.[127]

Although it appears that the above-mentioned urinary biomarkers perform well for the diagnosis of *established* AKI in adult patients,[128] their ability to *predict* AKI are

generally less robust. The predictive values have been inconsistent across studies for urinary HNL/NGAL (AuROC, 0.50–0.98),[129, 130] cystatin C (AuROC, 0.50–0.72),[129, 131] α_1-microglobulin (AuROC, 0.62–0.89),[129, 132] KIM-1 (AuROC, 0.68–0.78),[129, 133] IL-18 (AuROC, 0.53–0.89)[134, 135] and NAG (AuROC, 0.61–0.72).[133, 136] These biomarkers need further validation in patients with different critical conditions and co-morbidities before they can be regarded as clinically useful AKI predictors.

AIMS OF THE STUDY

The general aim was to investigate the performance of different biomarkers and their assays in predicting adverse outcomes, i.e. acute kidney injury and mortality, in critically ill patients and the impact of sepsis on biomarker levels. Our specific aims were:

1. To study the ability of plasma cystatin C to predict short- and long-term mortality in critically ill patients with and without AKI.

2. To study the impact of sepsis and AKI on HNL/NGAL levels in plasma and urine and to test whether the presence of septic shock affects the performance of plasma and urinary HNL/NGAL in predicting AKI.

3. To study the impact of sepsis on cystatin C levels in plasma and to investigate the predictive properties of plasma cystatin C for early detection of AKI, the need for acute RRT or mortality in ICU patients with and without sepsis.

4. To examine the ability of two ELISAs to detect HNL/NGAL released in urine from kidney epithelial cells and neutrophils respectively.

SUBJECTS AND METHODS

Table 2. Summary of subjects and methods used in Studies I–IV

	Study I	**Study II**	**Study III**	**Study IV**
Data source	PRONX, Population Register	PEAK	PEAK, PROTIVA, EXCRETe	PEAK
Design	Prospective cohort study	Case-control study	Prospective cohort study	Prospective cohort study
Study period	2003–2007	2007–2008	2007–2010	2007–2009
Participants (n)	AKI (271) Non-AKI (562)	Non-AKI SIRS (10) Severe sepsis (10) Septic shock (7) AKI Septic shock (18)	AKI-/sepsis- (151) AKI-/sepsis+ (80) AKI+/sepsis- (24) AKI+/sepsis+ (72)	Urinary HNL/NGAL ≥ 50 µg/l (47)
Exposure	Plasma cystatin C level	HNL/NGAL in urine or plasma	Sepsis *or* Plasma cystatin C on ICU admission	Monomeric/Dimeric HNL/NGAL in urine
Outcome	Long-term mortality	AKI	Cystatin C change *or* Sustained AKI, worsening AKI or mortality	ELISA-1 and ELISA-2 levels
Statistical analysis	Cox regression	ROC analysis	Repeated measures ANOVA, ROC analysis	Quantile regression

REGISTERS AND DATABASES

All databases exclusively include patients referred to the general ICU at the Karolinska University Hospital Solna.

The Total Population Register (Study I)

The register contains data from the Swedish census since 1968 and is managed by Statistics Sweden. The Swedish national registration number allows identification and follow-up of patients in the register with respect to short- and long-term mortality.

The PRONX Database (Study I)

All consecutive patients admitted to the ICU between June 2003 and November 2007 were screened for eligibility in the PRONX (PROspektiv Njurstudie på KS) database. Inclusion criteria were: (1) plasma creatinine > 150 µmol/l, (2) plasma urea > 25

mmol/l or (3) oliguria/anuria (urinary output < 800 ml/24 h or < 30 ml/h for 6 h). Patients treated with RRT were excluded. Plasma cystatin C and creatinine were measured at inclusion and patients were classified according to the RIFLE criteria. For comparison, we retrospectively included ICU patients who did not meet the predefined AKI criteria and had cystatin C measured on ICU admission between June 2006 and November 2007 (non-AKI cohort).

The PEAK Database (Studies II–IV)

Patients with a GFR > 60 ml/min/1.73m^2 on admission, estimated by the simplified MDRD formula,[7] and an expected ICU length of stay of more than 24 h were included in the Predicting Early Acute Kidney injury (PEAK) database. Study samples (blood and urine) were collected twice daily from admission until discharge or earlier if RRT was initiated. Patients were classified according to the RIFLE and AKIN criteria on a daily basis using both the creatinine and urinary output criteria (Table 1). If present, creatinine values obtained within 48 h before ICU admission, as well as 48 h after ICU discharge, were included in the RIFLE/AKIN classification. The presence (or absence) of systemic inflammatory response syndrome (SIRS), sepsis, severe sepsis or septic shock on each ICU day was recorded in the database (Table 3). Baseline characteristics, Acute Physiology And Chronic Health Evaluation (APACHE) II score, ICU diagnosis and ICU mortality were recorded. Information about co-morbid conditions and 30-day mortality was collected retrospectively from the hospital-based electronic case-record system. Physiological parameters (urinary output, arterial blood pressure), biomarker concentrations, body weight and information about corticosteroid and antimicrobial therapy obtained as a part of routine care procedures were recorded repeatedly during the ICU stay.

The PROTIVA Database (Study III)

All consecutive multi-traumatized patients referred to the ICU were recorded in the PROTIVA (PROTeiner på IVA) database. The same routine variables that were recorded in the PEAK database were also recorded in the PROTIVA database.

The EXCRETe Database (Study III)

Patients with a GFR < 60 ml/min/1.73m^2 on admission, estimated by the simplified MDRD formula, or patients in whom RRT was initiated in the ICU were included in the EXtracorporeal Clearance & REsidual renal function during rrT (EXCRETe) database. Patients with RRT treatment prior to ICU admission were excluded. Routine variables obtained during the ICU stay were recorded in the database in the same way as for the PEAK and PROTIVA databases.

SCORING METHODS

Severity of acute kidney injury

Urine output and plasma creatinine levels were recorded on a daily basis in the PEAK, PROTIVA and EXCRETe databases. The lowest creatinine level found within 3 months prior to ICU admission was used as baseline for the individual creatinine-based RIFLE/AKIN classification (Table 1). When no true pre-admission creatinine value was available, baseline creatinine was estimated by the MDRD equation using a low normal value for GFR (75 ml/min/1.73 m^2).

SIRS/sepsis scoring

The SIRS and sepsis classifications used in Studies II-IV are detailed in Table 3.[35]

Table 3. SIRS and sepsis scoring.

SIRS	≥ 3 of the following criteria: 1. Body temperature > 38˚C or < 36˚C 2. Heart rate > 90 beats/min 3. Respiratory rate > 20 breaths/min or $PaCO_2$ < 4.3 kPa 4. White blood cell count > 12 or < 4 x 10^9 cells/l
Sepsis	SIRS together with a suspected infection
Severe sepsis	Sepsis together with ≥ 1 of the following criteria: 1. PaO_2/FiO_2 ratio < 27 2. Urine output < 0.5 ml/kg/h during > 1 h 3. Platelet count < 80 x 10^9 cells/l or a > 50% decline over 3 days 4. Arterial pH ≤ 7.30 5. Base deficit ≥ 5 mmol/l in association with hyperlactatemia (> 3 mmol/l)
Septic shock	Sepsis together with hypotension defined as: 1. Systolic blood pressure < 90 mmHg or mean arterial pressure < 70 mmHg during > 1 h despite adequate fluid resuscitation* or 2. Need for vasopressor agents despite adequate fluid resuscitation*

$PaCO_2$, partial pressure of arterial carbon dioxide; PaO_2, partial pressure of arterial oxygen; FiO_2, fraction of inspired oxygen. *≥ 20 ml cristalloid/kg body weight or ≥ 10 ml colloid/kg body weight.

The modification of the SIRS criteria applied by the Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) study group was also applied by us, i.e. at least three (instead of two) out of four criteria had to be fulfilled.[137]

LABORATORY ASSAYS

Blood samples taken as a part of routine care were analysed at the Department of Clinical Chemistry, Karolinska University Hospital Solna. Study samples (PEAK database) were immediately centrifuged at 2,000 rpm at 4˚C for 10 min. The supernatant plasma and urine were aliquoted into cryovials and stored at -80˚C and were later analysed at the Department of Clinical Chemistry, Uppsala University Hospital, Uppsala, or by Diagnostics Development (Uppsala, Sweden). Plasma samples were analysed for HNL/NGAL, procalcitonin (PCT), C-reactive protein (CRP), myeloperoxidase (MPO) and cystatin C. Urine samples were analysed for HNL/NGAL, creatinine, cystatin C and α_1-microglobulin. Assay characteristics are described in detail below and in Table 4.

HNL/NGAL immunoassays

Radioimmuno assay (RIA)

HNL/NGAL in plasma (Study II) and urine (Studies II and IV) were quantified by RIA. 50 µl of plasma or urine was mixed with 50 µl of ^{125}I-labelled HNL/NGAL (diluted to 8 µg/l in a dilution buffer) and 50 µl of rabbit anti-HNL/NGAL polyclonal antibodies (diluted 1/3,800 in assay buffer) and incubated for 3 h at room temperature. Thereafter, 500 µl of solid phase cellulose suspension containing secondary antibodies (anti-rabbit IgG) were added and the incubation was continued for 1 h at 4˚C. HNL/NGAL-antibody complexes bound on anti-rabbit IgG coated cellulose were separated by centrifugation at 3,400 rpm for 15 min. After decantation, the radioactivity was measured in a gamma counter. The intra- and inter-assay coefficients of variation (CVs) were < 6% and < 10%, respectively. Expected normal HNL/NGAL levels were < 73.5 µg/l in plasma and < 141 ng/mg creatinine in urine.

Western blot

The different molecular forms of HNL/NGAL in urine were detected by Western blotting in Study IV. 20 µl of urine were applied to Nu-PAGE® 4–12% Bis-Tris Gel

(Invitrogen Corporation, USA). After exposure to sodium dodecyl sulphate (SDS) and electrophoresis, proteins were transferred to a Hybone-P polyvinylidene fluoride (PVDF) membrane (GE Healthcare, UK) using Nu-PAGE® transfer buffer at 25 V for 1 h. Additional binding sites of the PVDF membrane were blocked by a blocking solution (GE Healthcare, UK) for 1 h. Thereafter, the blots were incubated with rabbit anti-HNL/NGAL polyclonal antibodies for 1 h. Finally, the blots were incubated for 45 min with peroxidase-conjugated secondary antibodies (GE Healthcare, UK). Immunoblots were detected by enhanced chemiluminiscence.

Enzyme-linked immunosorbent assay (ELISA)

Microtiter plates (Nunc Maxsorp, Agogent, Denmark) were coated with a monoclonal anti-HNL/NGAL antibody (clone 763; Diagnostics Development, Uppsala, Sweden) at 4˚C overnight. Additional binding sites were blocked with carbonate-bicarbonate buffer (Invitrogen Corporation, UK) at 37˚C for 1 h. 100 µl of urine diluted in assay solution were added in duplicates and incubated for 2 h at room temperature. 100 µl of diluted monoclonal anti-HNL/NGAL antibodies (clones 754 or 765) were added and incubated at room temperature for 1 h, followed by incubation with 100 µl of diluted horseradish peroxidase-conjugated antibodies (GE Healthcare, UK) during another 1 h at room temperature. Finally, 100 µl of tetramethylbenzidine solution were added to visualize the enzyme reaction. Absorbance was measured by a microplate-reader (SPECTRAmax 250, GMI, Inc., USA).

Additional assays

Table 4 summarizes the additional biomarkers, and their assay characteristics, analysed in Studies I–IV.

Table 4. Additional biomarkers presented in Studies I–IV.

Biomarker	Study	Immunoassay	Analyser	Total CV%	Expected normal
Plasma					
Creatinine[1]	I–IV	Alkaline picrate colorimetry	LX/DxC 800[a]	4–9%	< 100 µmol/l (men) < 90 µmol/l (women)
Cystatin C[1]	I, III	Turbidimetric	LX/DxC 800[a]	7% (1.0 mg/l) 3.9% (3.4 mg/l)	< 0.99 mg/l
Cystatin C[2]	II	Turbidimetric	Architect Ci8200[b]	1.7% (0.77 mg/l) 1.1% (1.25 mg/l)	< 1.55 mg/l (>50 yr) < 1.20 mg/l (<50 yr)
CRP[2]	II	Turbidimetric	Architect Ci8200[b]	4%	< 5 mg/l
CRP[1]	III	Turbidimetric	LX/DxC 800[a]	4–5%	< 3 mg/l
PCT[2]	II	ELISA	Cobas EE[c]	6% (0.25 µg/l) 3% (10.4 µg/l)	< 0.05 µg/l
MPO[3]	II	ELISA	SPECTRA-max 250[d]	< 6%	< 55.4 µg/l
Urine					
Creatinine[2]	II	Alkaline picrate colorimetry	Architect Ci8200[b]	5% (56 µmol/l) 3% (370 µmol/l)	2.5–16.4 mmol/24 h
Cystatin C[2]	II	Turbidimetric	Architect Ci8200[b]	2.96% (0.13mg/l) 2.67% (0.9 mg/l)	< 6.2 mg/g creatinine
α_1-microglobulin[2]	II	Turbidimetric	Architect Ci8200[b]	6% (36 mg/l) 9% (48 mg/l)	< 6.2 mg/g creatinine

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STUDY I

Design and study population

A total of 271 patients entered the AKI cohort in the PRONX database and 562 patients were included in the non-AKI cohort. 124 non-AKI patients had creatinine > 100 μmol/l or urea > 20 mmol/l and were classified as having a 'potential' AKI. The AKI cohort was stratified into four quartiles according to their cystatin C and creatinine levels at inclusion, i.e. at the time when the AKI criteria were met. AKI patients were also categorized into four groups based on their RIFLE stage (zero, R, I or F) at inclusion. The non-AKI patients were first divided into four quartiles according to their cystatin C level on ICU admission. Secondly, the patients in the highest quartile were divided into two separate groups. Information about long-term mortality was obtained from the Population Register.

Statistical analysis

The association between cystatin C strata and long-term mortality was estimated by hazard ratios (HRs) derived from the Cox proportional hazards regression model before and after adjustment for covariates. Cumulative survival curves were generated using the Kaplan-Meier methodology and differences between survival curves were examined using the log-rank test.

STUDY II

Design and study population

Sixty-five patients were included in the PEAK database between August 2007 and November 2008 and were assessed for eligibility in Study II. Non-AKI patients were categorized according to their worst SIRS/sepsis score in the ICU into: (1) SIRS (n = 10), (2) severe sepsis (n = 10) or (3) septic shock (n = 7). For comparison, we included a fourth category comprising AKI patients with septic shock (n = 18). Peak levels in plasma for HNL/NGAL, MPO, PCT, CRP, cystatin C and creatinine and in urine for HNL/NGAL, α_1-microglobulin and cystatin C were compared between the four categories. Urinary creatinine was measured and served to correct the urinary biomarker levels for variations in urine dilutions. HNL/NGAL levels in plasma and urine measured at 12 h before the time-point when AKI was first diagnosed (AKI day

0) were compared with the HNL/NGAL concentrations obtained from the fifth consecutive plasma and urine sample in non-AKI patients.

Statistical analysis

The Kruskal-Wallis test was used for overall comparisons of peak biomarker levels between the four categories. A *post-hoc* comparison between two categories was made using the Mann-Whitney test. The performance of HNL/NGAL in plasma and urine in predicting AKI within 12 h was assessed by calculating sensitivity, specificity and the AuROC. Optimal cut-off levels were obtained by visual inspection of the AuROCs, giving equal weight to sensitivity and specificity.

STUDY III

Design and study population

In Study III we included 327 patients registered in the PEAK, PROTIVA and EXCRETe databases between February 2007 and May 2010. AKI was defined as a rise in plasma creatinine of ≥ 50% relative to baseline. Patients were allocated to four different categories according to the presence of AKI and/or sepsis during the first week in the ICU: Category A, neither sepsis nor AKI (n = 151); Category B, sepsis without AKI (n = 80); Category C, AKI without sepsis (n = 24) and Category D, sepsis and AKI (n = 72). Our intention was to investigate the impact of the conditions AKI and sepsis on cystatin C levels in plasma. Patients may go in and out of these conditions during the ICU course. Furthermore, AKI and sepsis might not occur simultaneously in individual patients. To account for this, we only included variables on days when the predefined criteria for each category were satisfied. For patients in category D, for instance, we excluded recordings obtained on days when AKI and sepsis criteria were not satisfied on the same day. Changes in creatinine, cystatin C, CRP and body weight were compared between septic and non-septic patients over the study period of seven days. Cystatin C was correlated to CRP on each day. The performance of cystatin C on admission to predict sustained AKI (> 3 d), worsening AKI (increase in RIFLE stage or RRT initiation) or death within seven days was assessed separately in septic and non-septic patients.

Statistical analysis

Changes in creatinine, cystatin C, CRP and body weight over time were analysed using repeated measures analysis of variance (ANOVA) after logarithmic transformation (base 10). For comparison of changes over time between categories, an interaction variable (between category and time) was introduced in the ANOVA model. The relationship between CRP and cystatin C was measured by Spearman's rank correlation. Diagnostic characteristics of cystatin C were assessed by ROC analysis. AuROCs were compared using the χ^2-test.

STUDY IV

Design and study population

A total of 782 urine samples were obtained from 83 patients and included in the PEAK database between August 2007 and April 2009. All samples were analysed for HNL/NGAL using the RIA method in a first step. Urine samples with a RIA-measured HNL/NGAL concentration $\geq$ 50 μg/l were selected for further analysis by Western blotting and two different ELISAs (ELISA-1 and ELISA-2). This cut-off point was chosen due to limitations in the sensitivity of the Western blot procedure. The antibody configurations of the ELISAs were as follows: in both assays the microtiter plates were coated with the monoclonal antibody clone 763. The detecting antibodies in ELISA-1 and ELISA-2 were the monoclonal antibody clones 764 and 765, respectively.

The Western blot patterns were evaluated in two ways. By scanning of the electropherograms, relative relations between monomeric and dimeric HNL/NGAL were constructed (monomer/dimer ratio). By visual inspection of the blot patterns, urine samples were classified according to the presence of monomeric and/or dimeric HNL/NGAL into (Figure 4): mainly monomeric HNL/NGAL (Class 1), monomeric and dimeric HNL/NGAL (Class 2) or mainly dimeric HNL/NGAL (Class 3).

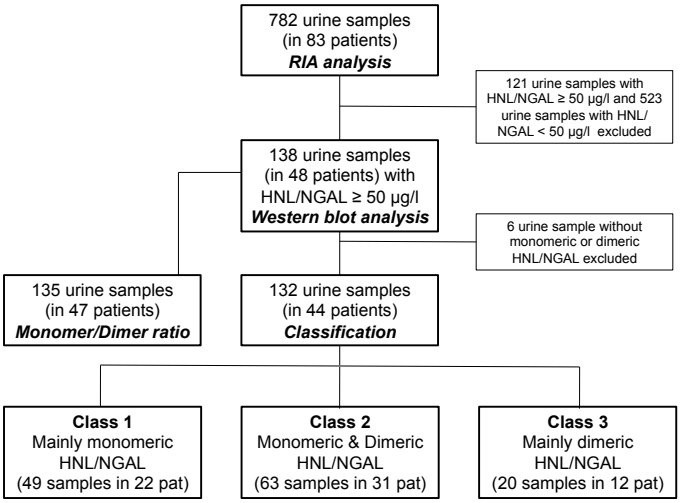

Figure 4. Selection of urine samples and classification according to urine sample results.

The ability of ELISA-1 and ELISA-2 to detect monomeric and dimeric HNL/NGAL was investigated by testing the association with the monomer/dimer ratio and by comparing the ELISA-1 and ELISA-2 concentrations between Classes 1–3. Finally, we identified patients with AKI as defined by a ≥ 50% increase in plasma creatinine from baseline or as an absolute rise in plasma creatinine of ≥ 26.4 µmol/l within 48 h according to the RIFLE/AKIN criteria. HNL/NGAL was quantified by ELISA-1 and ELISA-2 on the urine samples obtained from 24 h before (AKI day -1) until 48 h after (AKI day 2) the time-point when AKI was first diagnosed (AKI day zero).

Statistical analysis

The association between the monomer/dimer ratio and HNL/NGAL concentrations measured by ELISA-1 and ELISA-2, respectively, were investigated using quantile regression. We considered the 25^{th}, 50^{th} (median) and 75^{th} percentile. We also applied quantile regression for comparison of HNL/NGAL values between categories, using Classes 1, 2 and 3 as dummy variables in the regression model. The Wald test was used to compare the coefficients in the model. The median change over time for the ELISA-1 and ELISA-2 results was also tested by quantile regression using AKI day as the repeated-measures variable. Continuous variables were introduced in the statistical analyses after logarithmic transformation (base 10). Potential intra-individual dependence was taken into account in the regression models. Standard errors for the regression coefficients were obtained by generating 500 cluster-bootstrap samples, in which the individual was the re-sampling unit.

RESULTS

STUDY I

The AKI cohort

Figure 5 shows the survival curves for AKI patients divided according to cystatin C quartiles and RIFLE stages at inclusion. The hazard ratios describing the association between quartiles of cystatin C and mortality were not significant after adjusting for age, ICU diagnosis and RIFLE stage in the Cox regression model. However, Figure 5 indicates that cystatin C above the median (> 2.35 mg/l) was associated with higher long-term mortality.

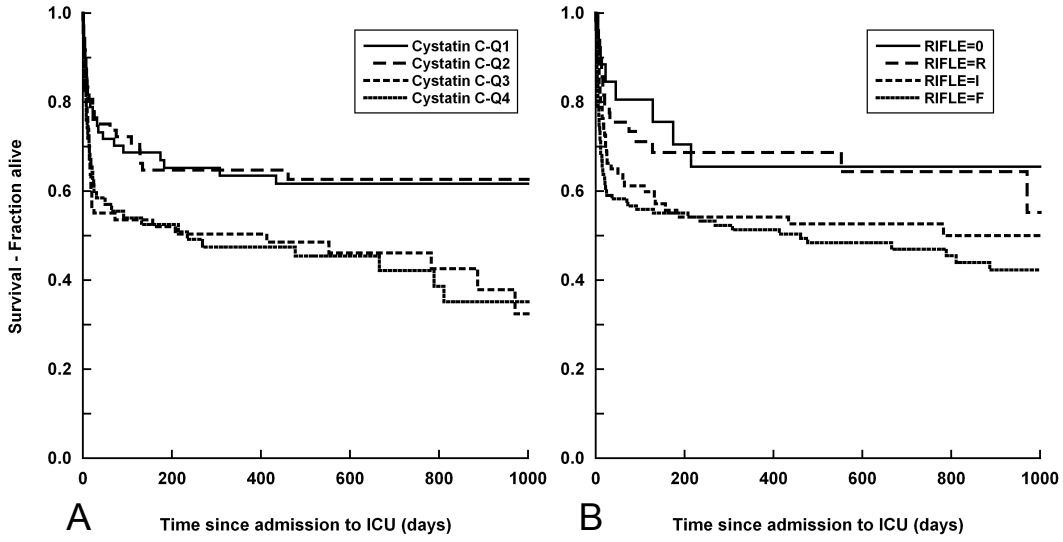

Figure 5. Survival curves of the AKI cohort by cystatin C (**A**) and RIFLE (**B**) level.

In fact, when we compared cystatin C above and below the median during the second year onwards, hazard ratios were significant even after adjusting for RIFLE stage and APACHE II score (Table 5).

Table 5. Relative risk of all-cause mortality in AKI patients

Cystin C level (mg/l)	**HR[a]**	**HR[b]**
Follow-up until day 365		
≤ 2.35	1.0 (ref)	1.0 (ref)
> 2.35	1.62 (1.10–2.38)	1.46 (0.99–2.16)
Follow-up from day 366 and onwards		
≤ 2.35	1.0 (ref)	1.0 (ref)
> 2.35	5.01 (1.31–19.21)	5.29 (1.37–20.39)

Hazard ratios (HRs) presented with 95% confidence intervals

[a]Adjusted for age, ICU diagnosis. [b]Adjusted for age, ICU diagnosis, RIFLE and APACHE II score.

The non-AKI cohort

In Figure 6 we show the survival curves of the non-AKI cohort stratified into quartiles based on cystatin C on ICU admission. Note that the highest quartile (Q4) was further divided into two groups (Q4:1 and Q4:2).

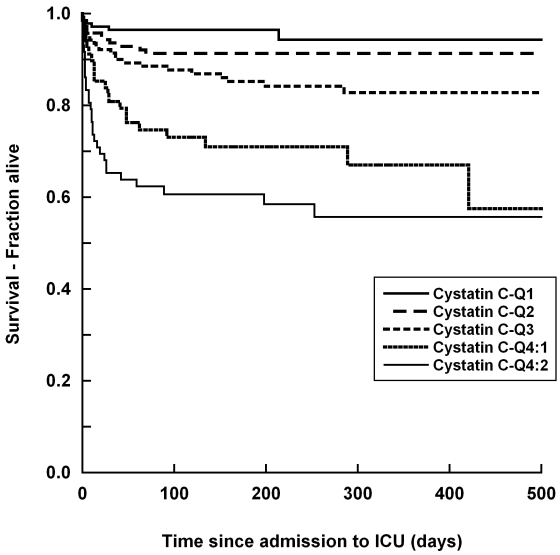

Figure 6. Survival curves of the non-AKI cohort by cystatin C level.

The point estimates for the relative risk of death increased with the cystatin C level and reached significance in the highest quartile (Q4). Higher point estimates were seen in the highest quartile after exclusion of 124 patients with potential AKI or when the analysis was restricted to patients under the age of 55 (Table 6).

Table 6. Relative risk of all-cause mortality in non-AKI patients

Cystatin C level (mg/l)	**HR[a]**	**HR[b]**	**HR[c]**
≤ 0.70 (Q1)	1.0 (ref)	1.0 (ref)	1.0 (ref)
0.71–0.91 (Q2)	1.78 (0.66–4.77)	1.81 (0.61–5.38)	1.39 (0.37–5.28)
0.92–1.40 (Q3)	2.22 (0.86–5.75)	3.01 (1.06–8.53)	1.20 (0.28–5.04)
1.41–1.78 (Q4:1)	3.81 (1.45–10.05)	5.53 (1.80–16.99)	6.15 (1.58–24.01)
> 1.78 (Q4:2)	5.74 (2.20–14.96)	5.95 (1.83–19.36)	12.60 (3.25–48.87)

Hazard ratios (HR) presented with 95% confidence intervals. Q, quartile.

[a]Adjusted for age, ICU diagnosis.

[b]Adjusted for age, ICU diagnosis and restricted to the 438 patients without potential AKI.

[c]Adjusted for age, ICU diagnosis and restricted to the 325 patients under the age of 55.

STUDY II

Biomarker levels in non-AKI patients

The three groups were equally distributed regarding APACHE II score. Length of stay in the ICU increased with sepsis severity. Peak creatinine levels and maximum changes in creatinine relative to baseline did not differ significantly between the three groups. CRP, PCT and MPO levels, reflecting the degree of systemic inflammatory response and neutrophil activation, increased with sepsis severity (Table 7). Peak levels of plasma HNL/NGAL increased with sepsis severity and were elevated in 22 of the 27 patients. Urinary HNL/NGAL levels also increased with sepsis severity but only four patients had peak urinary HNL/NGAL above the upper reference limit. Furthermore, a gradual increase in plasma and urinary cystatin C as well as urinary α_1-microglobulin was observed.

Table 7. Clinical characteristics and peak biomarker levels in the plasma and urine of patients without AKI

	SIRS (n = 10)	**Severe sepsis** (n = 10)	**Septic shock** (n = 7)
Age, yrs (IQR)	40 (30)	57 (11)	40 (46)
Male gender, n (%)	6 (60)	8 (80)	4 (57)
APACHE II score (IQR)	15 (15)	15 (6)	17 (6)
ICU length of stay, days (IQR)	3.0 (2.0)	4.5 (2.0)[a]	10 (9.0)[a, b]
True baseline creatinine available, n (%)	6 (60)	7 (70)	5 (71)
Peak biomarker levels in plasma			
HNL/NGAL (ng/ml)	111 (67.8)	116 (27.6)	134 (72.7)[b]
MPO (ng/ml)	108 (34.0)	198 (115)[a]	216 (502)[a]
PCT (ng/ml)	0.54 (1.3)	1.4 (2.8)	2.8 (10.3)[a]
CRP (mg/l)	112 (126)	251 (120)[a]	185 (137)[a]
Cystatin C (mg/l)	0.8 (0.3)	1.0 (0.5)	1.4 (0.4)[a]
Creatinine (μmol/l)	93.5 (29.0)	84.5 (21.0)	94.0 (52.0)
Creatinine change relative to baseline (%)	12.9 (27.6)	2.1 (28.4)	22.7 (18.7)
Peak biomarker levels in urine			
HNL/NGAL (ng/mg creatinine)	24.4 (34.4)	47.7 (29.1)	63.5 (133)[a]
α_1-microglobulin (mg/g creatinine)	96.6 (78.1)	138 (111)	159 (155)
Cystatin C (mg/g creatinine)	0.35 (0.22)	0.86 (1.86)[a]	1.73 (7.31)[a]

Values are expressed as the median (interquartile range [IQR]) or as n (%). SIRS, systemic inflammatory response syndrome; AKI, acute kidney injury.

[a] $p < 0.05$ compared with SIRS.

[b] $p < 0.05$ compared with severe sepsis.

Biomarker levels in AKI and non-AKI patients with septic shock

CRP, PCT and MPO levels did not differ significantly between septic shock patients with and without AKI, indicating that the sepsis-induced inflammatory response was comparable between these two groups. Additionally, there was no significant difference in peak plasma HNL/NGAL levels between septic shock patients with and without AKI. Of the three potential markers of acute tubular injury, only levels of HNL/NGAL in urine were significantly higher in the AKI cohort. Urinary HNL/NGAL was a good predictor of AKI occurring within 12 h. In plasma, HNL/NGAL performed less well as an AKI predictor (Table 8).

Table 8. Diagnostic characteristics of HNL/NGAL in plasma (p) and urine (u) for predicting AKI within 12 h in patients with septic shock.

Variables	Cut-off value	AuROC	Sensitivity	Specificity
pHNL/NGAL	> 120 µg/l	0.67 (0.39–0.94)	0.83 (0.36–1.0)	0.50 (0.12–0.88)
uHNL/NGAL	> 68 ng/mg creatinine	0.86 (0.68–1.0)	0.71 (0.29–0.96)	1.0 (0.54–1.0)

STUDY III

Non-AKI patients with and without sepsis

Characteristics of non-AKI patients with and without sepsis are compared in Table 9.

Table 9. Characteristics of non-AKI patients with and without sepsis.

	No sepsis (n = 151)	**Sepsis** (n = 80)	*p* value
Male sex, n (%)	115 (76)	64 (80)	0.5
Age (years)	41 (31)	41 (33)	1.0
APACHE II score	13 (8)	16 (9)	0.0004
True baseline creatinine available, n (%)	116 (77)	59 (74)	0.6
Admission creatinine, µmol/l	79 (26)	83 (29)	0.04
Admission cystatin C, mg/l	0.72 (0.23)	0.80 (0.47)	0.04
Admission body weight, kg	78 (20)	78 (28)	0.7
Corticosteroid treatment, n (%)	12 (8)	18 (23)	0.002
High dose steroids*, n (%)	6 (4.0)	3 (3.8)	1.0
Outcomes			
ICU length of stay, days	2.4 (1.9)	6 (5.9)	0.0001
ICU mortality, n (%)	4 (2.6)	3 (3.8)	0.7
30-day mortality, n (%)	6 (4.0)	6 (7.5)	0.3

Values are expressed as the median (IQR) or as n (%).

*Daily dose of > 300 mg hydrocortisone or equivalent.

Septic patients had higher APACHE II scores, longer ICU lengths of stay and were more often treated with corticosteroids, at least in low doses. Cystatin C and creatinine concentrations on admission were slightly higher in the septic group.

Mean changes in cystatin C, creatinine and body weight over the first week in the ICU are displayed in Figure 7. Cystatin C increased and creatinine decreased significantly in septic ($p = 0.0001$) and non-septic patients ($p < 0.0001$) during the study period. The observed changes did not differ significantly between patients with and without sepsis ($p = 0.59$ for cystatin C and $p = 0.78$ for creatinine). Significant variations in body weight were only seen in the septic category and reached a 0.8% increase on the average by day 3.

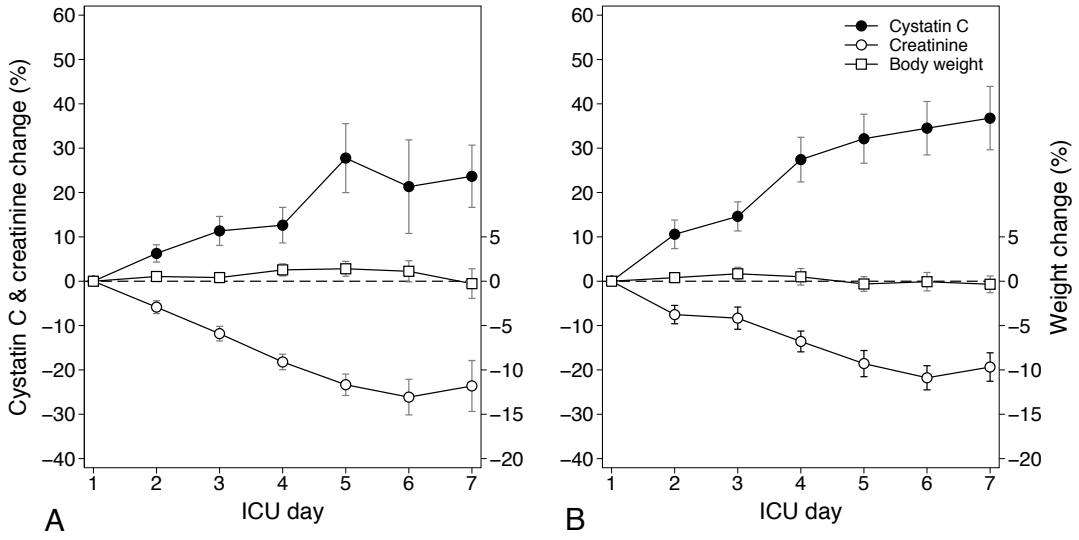

Figure 7. Change (mean ± SEM) in cystatin C (closed circles), creatinine (open circles) and body weight (open squares) relative to admission values: (**A**) non-AKI patients without sepsis; (**B**) non-AKI patients with sepsis.

AKI patients with and without sepsis

Characteristics of the AKI patients with and without sepsis are outlined in Table 10. Patients with septic AKI were older and stayed longer in the ICU than non-septic patients. The proportion of patients treated with RRT and corticosteroids was higher in the septic cohort.

Table 10. Characteristics of AKI patients with and without sepsis

	No sepsis (n = 24)	**Sepsis** (n = 72)	*p* value
Male sex, n (%)	17 (71)	51 (71)	1.0
Age (years)	55 (33)	64 (18)	0.08
APACHE II score	18 (16)	22 (9)	0.2
True baseline creatinine available, n (%)	12 (50)	39 (54)	0.7
Admission creatinine, µmol/l	115 (53)	121 (83)	0.9
Admission cystatin C, mg/l	1.21 (0.58)	1.36 (0.82)	0.2
Admission body weight, kg	87 (27)	80 (28)	0.9
Corticosteroid treatment, n (%)	4 (17)	28 (39)	0.08
High dose steroids*, n (%)	1 (4.2)	9 (12.5)	0.4
Outcomes			
RRT, n (%)	4 (17)	27 (38)	0.08
ICU length of stay, days	3.5 (2.5)	10 (10)	0.0001
ICU mortality, n (%)	3 (12.5)	9 (12.5)	1.0
30-day mortality, n (%)	5 (21)	15 (21)	1.0

Values are expressed as the median (IQR) or as n (%).
*Daily dose of > 300 mg hydrocortisone or equivalent.

Cystatin C gradually increased ($p < 0.0001$) during the first week in the septic AKI patients. By day 7, cystatin C levels had almost doubled on average (Figure 8). During the same time frame, creatinine did not change significantly ($p = 0.86$). The number of patients in the non-septic cohort was low and cystatin C results were only available during the first five days. During this period, cystatin C-changes did not differ significantly between septic and non-septic AKI patients ($p = 0.13$). Body weight changed significantly in both the septic ($p = 0.0001$) and non-septic patients ($p = 0.02$).

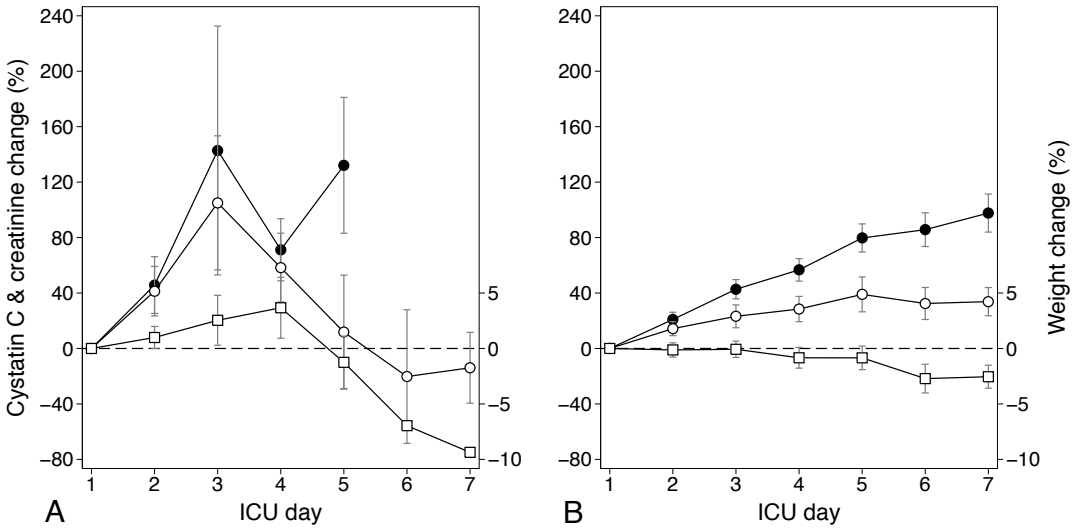

Figure 8. Change (mean ± SEM) in cystatin C (closed circles), creatinine (open circles) and body weight (open squares) relative to admission values: (**A**) AKI patients without sepsis; (**B**) AKI patients with sepsis.

Inflammatory response in septic and non-septic patients

In study III we assumed that the sepsis-induced systemic inflammation is more severe than systemic inflammation induced by other causes (e.g. trauma). To investigate this, we used CRP as an indicator of systemic inflammation for comparisons between septic and non-septic patients. On ICU admission, mean CRP levels were more than 4-fold higher in septic patients than in non-septic patients ($p < 0.0001$). CRP decreased in septic patients and increased in non-septic patients during the study period. Still, CRP levels remained significantly higher in septic patients during the first five days (Figure 9). Furthermore, we found no significant correlation between CRP and cystatin C on any of the seven days.

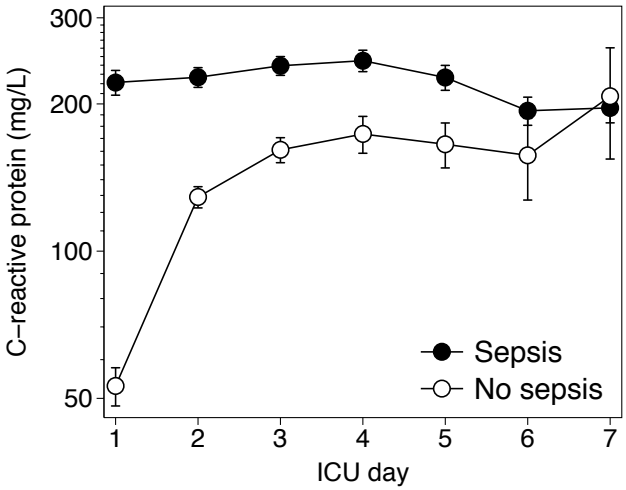

Figure 9. CRP levels (mean ± SEM) in septic (closed circles) and non-septic (open circles) patients.

Impact of sepsis on cystatin C as a predictor of adverse events

Sixty-seven patients with and 262 patients without sepsis on ICU admission had existing admission values of cystatin C. ROC curves for cystatin C as a predictor of sustained AKI, worsening AKI or death are shown in Figure 10. Cystatin C predicted the composite outcome in non-septic patients with an AuROC of 0.78 (95% CI, 0.70–0.85). For septic patients, the corresponding AuROC was 0.80 (95% CI, 0.68–0.91). No significant difference between the two AuROCs was found ($p = 0.76$).

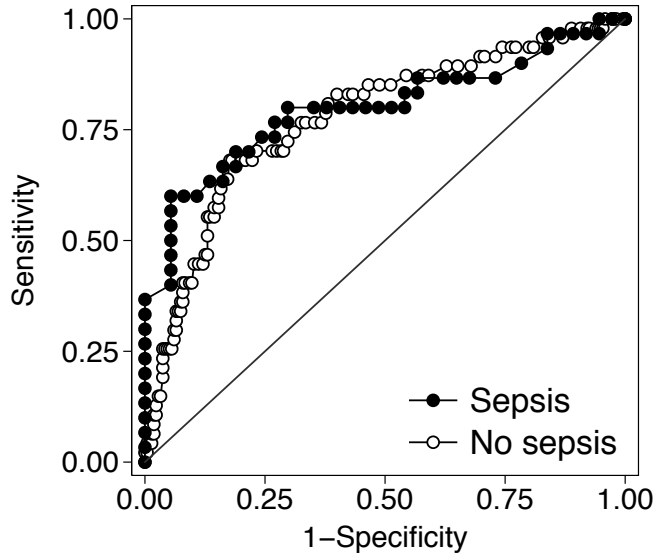

Figure 10. Receiver operating characteristics curves for cystatin C on ICU admission as a predictor of worsening AKI, sustained AKI or mortality within 7 days in septic (closed circles) and non-septic (open circles) patients.

STUDY IV

Association between HNL/NGAL levels and the monomer/dimer ratio

Figure 11A-C shows the association between the relative amount of monomeric HNL/NGAL (monomer/dimer ratio) and HNL/NGAL quantified by the RIA, ELISA-1 and ELISA-2 across the 25th, 50th (median) and 75th percentiles. HNL/NGAL quantified by the RIA and ELISA-1 increased with an increasing monomer/dimer ratio, whereas ELISA-2 results decreased with an increasing monomer/dimer ratio. The relationships were, however, only significant for the 50th and 75th percentiles. The ELISA-1/ELISA-2 ratio showed a stronger association with the relative amount of monomeric HNL/NGAL which was significant across all three percentiles (Figure 11D).

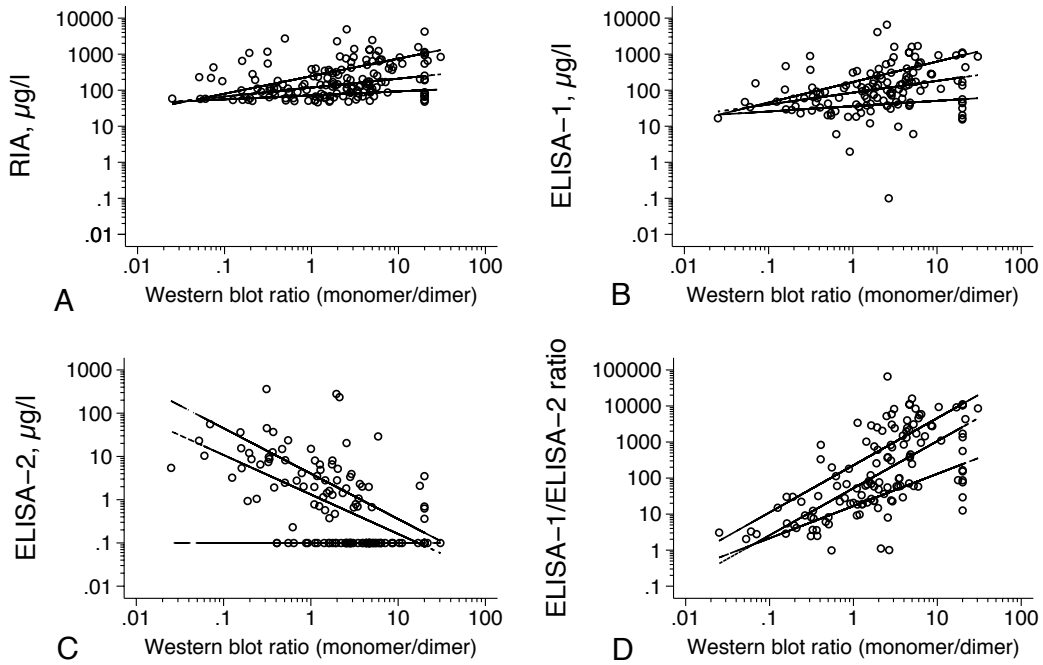

Figure 11. Scatter plots showing the association between the monomer/dimer ratio and HNL/NGAL measured by RIA (**A**), ELISA-1 (**B**), ELISA-2 (**C**) and the ELISA-1/ELISA-2 ratio (**D**), respectively. Superimposed on the plots are the fitted 25th, 50th (median) and 75th percentile regression lines.

Assay levels in samples with monomeric and/or dimeric HNL/NGAL

HNL/NGAL levels quantified by the RIA, ELISA-1, ELISA-2 and the ELISA-1/ELISA-2 ratio in samples visually classified as containing monomeric and/or dimeric HNL/NGAL are displayed in Figure 12 and summarized in Table 11. The median ELISA-1 concentration was significantly higher in samples containing mainly monomeric (Class 1) as compared to mainly dimeric (Class 3) HNL/NGAL. The opposite was true for ELISA-2. Finally, the median ELISA-1/ELISA-2 ratio was almost 240 times higher in Class 1 than Class 3 with the category containing both monomeric and dimeric HNL/NGAL as an intermediate.

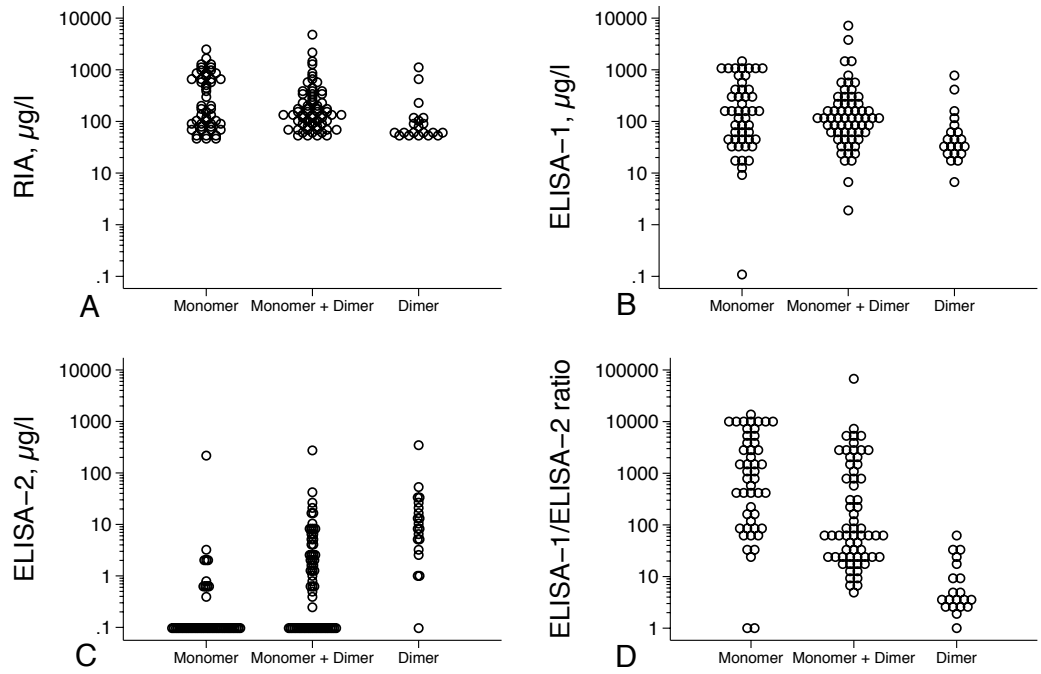

Figure 12. HNL/NGAL levels quantified by by RIA (**A**), ELISA-1 (**B**), ELISA-2 (**C**) and the ratio between ELISA-1 and ELISA-2 (**D**) in urine samples classified as containing mainly monomeric HNL/NGAL (Monomer, Class 1), monomeric and dimeric HNL/NGAL (Monomer + Dimer, Class 2) or mainly dimeric HNL/NGAL (Dimer, Class 3).

Table 11. Median HNL/NGAL levels measured by RIA, ELISA-1, ELISA-2 and the ELISA-1/ELISA-2 ratio in the different Western blot classes

	Class 1 (Monomer)	**Class 2** (Monomer+Dimer)	**Class 3** (Dimer)	Overall *p* value†
Median RIA, µg/l (IQR)	169.6 (606.88)	143 (231.7)[b]	63.78 (56.38)	< 0.001
Median ELISA-1, µg/l (IQR)	144.99 (388.44)[b]	118.07 (183.82)[b]	45.4 (64.49)	0.0128
Median ELISA-2, µg/l (IQR)	0.1 (0)[a, b]	0.704 (4.01)[b]	10.37 (18.31)	< 0.001
Median ELISA-1/ELISA-2 ratio (IQR)	959.82 (4217)[a, b]	67.55 (1002)[b]	4.127 (6.359)	< 0.001

†*p* values refer to the overall comparison of medians between Classes 1–3 using quantile regression and Wald test statistics, accounting for intra-individual dependence.
[a]$p < 0.05$ compared with Class 2, [b]$p < 0.05$ compared with Class 3.

Panel of HNL/NGAL assays during AKI development

Figure 13A displays the kinetics of HNL/NGAL, quantified by the ELISA-1 as well as the ELISA-2, in 32 AKI patients, of which 23 (78%) had sepsis, during the time frame of 24 h before and up to 48 h after the AKI diagnosis. In Figure 13B, the ELISA-1/ELISA-2 ratios during the same time frame is shown.

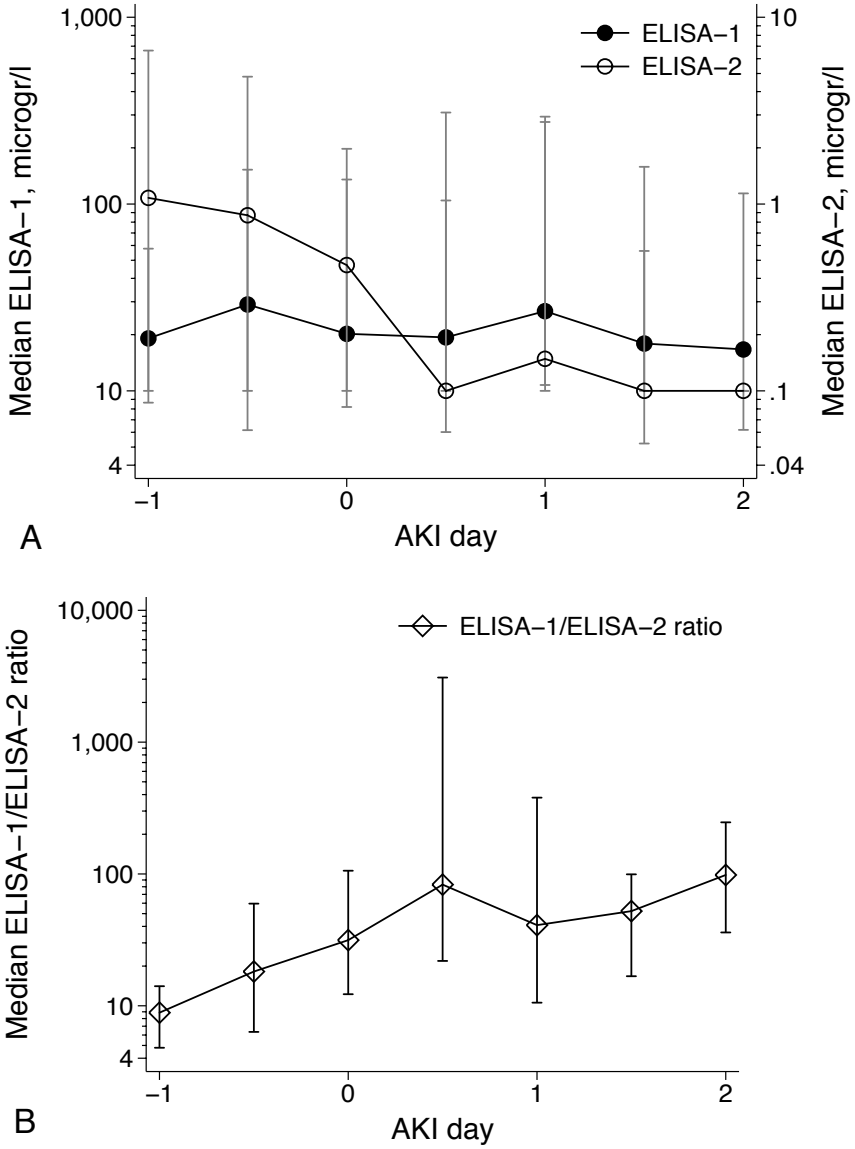

Figure 13. Median HNL/NGAL levels quantified by the ELISA-1 (**A**, closed circles) and ELISA-2 (**A**, open circles) and the median ELISA-1/ELISA-2 ratios (**B**, hollow diamonds) during the time frame from 24 h before AKI (AKI day -1) and up to 48 h after the AKI diagnosis (AKI day 2).

DISCUSSION

METHODOLOGICAL CONSIDERATIONS

Study design

The information about relevant exposures and outcomes, such as sepsis and AKI, was assessed prospectively in the databases generating the study populations in Studies I–IV. Cohort studies are preferred when the outcome of interest is frequent. Conversely, when a rare outcome is expected, a case-control design is preferable.[138] As stated above, AKI is a frequent outcome in the ICU. However, 20–40% of these patients already have AKI (according to RIFLE) on ICU admission.[20, 139, 140] Since the main purpose of the PEAK database was to study biomarkers for AKI *prediction,* we only included patients with apparently normal renal function (GFR > 60 ml/min/1.73m^2) on admission. Therefore, the frequency of AKI is lower in the PEAK database than expected in the general ICU population. To investigate the performance of HNL/NGAL in predicting future AKI (Study II), we used a nested case-control design. The predictive value for cystatin C was, on the other hand, evaluated using a cohort design in Study III. This was possible because *sustained* AKI (> 3 d) was part of the composite outcome and patients were therefore allowed to have AKI on admission.

Generalizability

The aim of many studies is to make an inference from the underlying source population from which the study sample is drawn. Since Studies I–IV are all single-centre studies, our findings may not be applicable to other ICUs treating a different case-mix. Generalizability is further reduced by our eligibility criteria. Studies II and IV exclude patients with renal dysfunction on admission. Study III included patients from three databases, consisting of different sub-populations, with overrepresentation of trauma patients from the PROTIVA database. Trauma patients are generally younger and have less frequent co-morbid conditions than the general ICU population. Study I is more representative since all consecutive patients were included in the AKI and non-AKI cohorts. In Study IV we only included urine samples with an HNL/NGAL concentration above 50 µg/l. Therefore, we cannot be sure that our assays effectively distinguish monomeric from dimeric HNL/NGAL at lower concentrations.

Misclassification

Misclassification of AKI

To investigate the performance of HNL/NGAL in predicting acute kindney *injury*, we used the RIFLE and/or AKIN classification as the reference method. This is problematic for two reasons. First, changes (absolute or relative) in plasma creatinine or urine output are markers of kidney function (i.e. GFR) rather than markers of renal cell damage. Increased creatinine levels and/or decreased urine output can be a physiological response to hypovolaemia/hypotension without any signs of kidney injury. Patients in whom such responses occur will certainly be misclassified as having AKI. The opposite may also be true – damage to the kidney epithelium might go undetected by changes in creatinine and urine output. Second, the RIFLE/AKIN criteria are based on changes in creatinine from baseline. When baseline creatinine is unknown, which may often be the case, it is estimated by the MDRD formula using a GFR in the lower normal range (75 ml/min/1.73m^2). This might overestimate baseline creatinine (and hence underestimate the relative creatinine change), especially in patients with habitually low creatinine levels due to reduced muscle mass, with the risk of misclassifying AKI patients into the non-AKI cohort. Conversely, in patients with unknown CKD, the baseline will be underestimated and these patients will be misclassified as having AKI.[141]

Misclassification cannot be ruled out in Study II where more non-AKI patients (29–40%) had missing baseline values as compared to the AKI patients (22%). In Study III a true baseline was only present in 50% of AKI patients and in as many as 70% of non-AKI patients. Our comparison between patients with and without sepsis was, however, restricted to the AKI and non-AKI cohorts, respectively. Fortunately, the same proportion of available baseline values was present in septic and non-septic patients.

Misclassification of sepsis

The SIRS criteria describe the physiological response to non-infectious triggers such as trauma, pancreatitis and immunological reactions. Unfortunately, the criteria are overly sensitive and hence describe a heterogeneous group of disorders with different causes and outcomes. When SIRS arises in response to a *suspected* infection, sepsis is said to be present. Often, an infection is difficult to confirm microbiologically and patients

with 'pure' SIRS might be misclassified as having sepsis. The definitions of severe sepsis and septic shock are also non-specific, especially when they are used to describe different stages of severity. In fact, patients with septic shock do not always satisfy the criteria for severe sepsis. One of our aims in Study II was to investigate biomarker levels in patients with SIRS, severe sepsis and septic shock. Here, we assumed that the systemic inflammatory response was least severe in the SIRS group and most severe in patients with septic shock. This might not always be the case: a patient with SIRS can be in circulatory shock with severe multiorgan failure but without any signs of an infection, whereas a patient classified as having septic shock may simply have a slow infusion of a vasopressor without other signs of organ failure. Luckily, our biomarkers of inflammation (CRP, MPO) and infection (PCT) supported an increasing inflammatory response going from SIRS to septic shock.

Confounding

Confounders are factors that are associated with both the putative cause and its effect. In contrast, intermediate factors in the causal pathway between exposure and outcome are not confounders. Confounders can be dealt with in several ways: in the study design by randomization, restriction and matching, and in the data analysis by stratification or by adjusting for the confounder in a regression analysis.

Study I

Multivariable regression was used in Study I to demonstrate the confounding effect of AKI severity (RIFLE stage) on the association between cystatin C levels and mortality in the AKI cohort. Illness severity (APACHE II), ICU diagnosis and age were also considered potential confounders and were therefore adjusted for in the analysis.

Studies II and III

Both restriction and stratification were used in Studies II and III to investigate the impact of sepsis on biomarker levels in plasma and urine. In Study II we *stratified* patients according to sepsis severity and *restricted* the analysis to non-AKI patients in order to remove the effect of AKI and demonstrate the impact of sepsis on biomarker levels. In the same study we also restricted our ROC analysis to patients with septic shock in order to assess the performance of HNL/NGAL in predicting AKI. In Study III

we restricted patients to AKI and non-AKI cases to investigate the impact of sepsis on cystatin C in these separate groups. In the next step we stratified patients according to the presence or absence of sepsis and compared the predictive performance of cystatin C between these two strata.

Random errors

The Latin word 'error' means 'to wander' and its use in statistics refer to fluctuations of measurements around the true value. A *systematic* error tends to shift all measurements away from the true value in a predictable and systematic way. An example is when an instrument is not correctly calibrated. In contrast to systematic errors, *random* errors are inherently unpredictable and will always be present when a biological quantity is being measured. The precision of a measurement is largely affected by random errors and can be reflected by the *p* value and the confidence interval. In this thesis we have compared biomarker levels between different pre-defined groups of ICU patients, drawn from a source population – mainly AKI and non-AKI patients with and without sepsis. We found that the median biomarker levels were not identical among these groups. Does this reflect the likelihood that a true difference exists between these groups in the source population *or* was it just a coincidence of random sampling? Since we do not have data on the entire source population, we can only say whether a random error is probable or not. The *p* value is a probability, which answers the question: If the populations really have the same median biomarker levels, what is the probability that random sampling would lead to a difference between sample medians as large (or larger) than we observed? In other words, if the *p* value is 0.01, for example, random sampling from identical populations would lead to a difference smaller than we observed in 99% of experiments and larger than we observed in 1% of experiments.

If we conclude that there is a difference between biomarker levels among our groups when, in fact, no difference exists, a type I error would occur. The probability of making a type I error is usually allowed to be less than 5% and is referred to as the *level* of the test.

If we, instead, conclude that there is no difference between our groups, a difference may still exist, but our sample is not large enough to detect it (type II error). This may

have happened in Study II when we failed do demonstrate significant differences in biomarker levels between some of the groups.

INTERPRETATION OF FINDINGS

So far, the diagnosis of AKI has relied upon markers of GFR (kidney *function*) instead of renal cell damage. There are reasons to believe that future treatment of AKI will depend on our ability to detect AKI as early as possible after the kidney insult, even before the functional impairment is obvious. To date, a number of potential markers of renal cell damage have emerged. However, their ability to detect AKI and monitor the course of the disease appears to be hampered by the confounding effect of other co-existing conditions. An ideal AKI biomarker should be:[142] (a) rapid and easy to measure in blood or urine; (b) sensitive in order to establish an early diagnosis while damage is still potentially reversible; (c) specific to AKI; (d) associated with a known mechanism; (e) should increase in proportion to the degree of damage; and (f) be able to monitor the course of AKI.

In addition, it should be pointed out that robust functional measurements of GFR will continue to be important, e.g. for drug dosing. In this thesis, the impact of sepsis on levels of cystatin C and HNL/NGAL in critically ill patients with and without AKI is investigated. An improved platform to detect different sources of HNL/NGAL in urine is also suggested and may be a useful tool as a monitor of pathophysiological changes during AKI development.

Cystatin C

In contrast to creatinine, cystatin C satisfies many features of an ideal filtration marker. In some studies cystatin C outperformed creatinine in detecting minor reductions in GFR.[143, 144] Furthermore, Shlipak et al. found that cystatin C predicted mortality in outpatients with apparently normal kidney function.[145] The practical use of cystatin C as a marker of kidney function and/or mortality in general ICU patients has not, however, been fully investigated.

The aim of Study I was to investigate whether cystatin C had the ability to predict long-term mortality, irrespective of the presence of AKI, in a general ICU population. In this

study we found that plasma cystatin C was independently associated with long-term mortality. We reached this conclusion by analysing our data in several steps. First, we observed a gradual increase in mortality among cystatin C quartiles in our AKI cohort. Significance was not reached, however, when we adjusted for AKI severity (RIFLE stage). This was expected since cystatin C mirrors reduced GFR, which is a feature of AKI, and because there is a well-known association between AKI severity and mortality.[146] Second, when we compared cystatin C above and below the median (2.35 mg/l) from the 2nd year and onwards, a strong association with mortality was found, independently of AKI severity. The corresponding association could not be demonstrated during the first year. This suggests that AKI has an early impact on mortality and that cystatin C carries additional information when this early risk has resolved. Third, when we investigated our non-AKI cohort we found an association between ICU admission levels of cystatin C and mortality. This association was further strengthened after removing patients with 'potential' AKI.

Our results raise the question: Do patients enter the ICU with differing baseline risks, dependent on or independently of GFR, and does cystatin C measure this risk? In the above-mentioned study by Shlipak et al., cystatin C predicted subsequent development of CKD in patients with a normal GFR (estimated by creatinine).[145] Moreover, Van Biesen et al. found that even mild reductions in kidney function in apparently healthy individuals are associated with increased mortality.[147] Perhaps CKD is the intermediate link between cystatin C and late mortality in our study. It should be possible to answer this question if ICU patients with different cystatin C values were followed a long time after ICU discharge with GFR measurements, using established reference methods (e.g. inulin or iohexol clearance).

An alternative explanation to our observed association is that non-renal factors affect the levels of cystatin C in plasma. Others have found a correlation between cystatin C levels and CRP[80, 81] as well as elevated levels in patients with HIV[148] and leukemia.[149] This may indicate a possible impact of inflammation on cystatin C. Cystatin C is a strong inhibitor of proteolytic enzymes, e.g. caspases. Since caspase-activity is up-regulated in sepsis,[37] we speculated that sepsis *per se* might trigger the increase in cystatin C. This was further investigated in Studies II and III.

In Study II we compared peak levels of plasma cystatin C between non-AKI patients with SIRS, severe sepsis and septic shock and found a gradual increase in peak levels with increasing sepsis severity. The additional analysis of several markers of inflammation (CRP, MPO, PCT) supported the view that the systemic inflammatory response increased with sepsis severity (Table 7). Furthermore, we compared cystatin C between septic shock patients with and without AKI. The inflammatory markers did not differ significantly between these two latter groups, but cystatin C was significantly higher in the AKI patients. Indeed, this study indicates an impact of sepsis on cystatin C levels and, as could be expected, that the levels increase further in AKI. However, the fact that septic patients also stayed longer in the ICU reflects that these patients were sicker and thus more extensively exposed to other known or unknown factors that may have a potential impact on the cystatin C levels.

In fact, this was illustrated in Study III in which we explored the impact of the sepsis-induced systemic inflammatory response on cystatin C alterations on each of the first seven days in the ICU. Interestingly, we found a daily increase in cystatin C in our ICU patients, even in those without AKI. But in contrast to what we had expected from the results in Study II, a similar increase was observed in both septic and non-septic patients. The assumption that sepsis per se does not affect cystatin C levels was further supported by our additional findings: (1) no correlation between cystatin C and CRP was found on any of the seven days and (2) the performance of cystatin C in predicting our composite outcome was similar in septic and non-septic patients. It is, however, possible that the systemic inflammatory response, irrespective of the presence of sepsis, was responsible for the increase in cystatin C. In fact, our non-septic cohorts mainly consisted of patients with SIRS. The fact that CRP levels were significantly higher in septic patients during the first five study days reflects that sepsis induced a more severe inflammatory response. Furthermore, Grubb et al. found no changes in cystatin C levels after surgically induced systemic inflammation.[82]

Then what does this daily increase reflect? Is it a gradual decline in GFR? This is unlikely since creatinine gradually decreased during the same time frame. There are, however, several reasons for why creatinine does not accurately reflect early changes in GFR. Increased TBW due to massive volume expansion can dilute plasma

creatinine.[150] This is not a plausible explanation for the observed changes in our study since only minor variations in body weight were observed, at least in the non-AKI patients. Any significant changes in TBW in these patients are therefore highly unlikely. A more plausible explanation could be decreased creatinine production, e.g. due to a gradual loss of muscle mass, which is a well-known phenomenon in ICU patients.

It is also a well-known fact that corticosteroids induce the production of cystatin C, at least in high doses.[67, 151] Furthermore, it appears that steroids affect cystatin C and creatinine in opposite directions.[68] Theoretically, this could have some impact on the different changes seen in Study III. It is, however, unlikely that the low proportion of patients treated with high-dose steroids caused the divergent changes in creatinine and cystatin C over time. In the light of the drawbacks of creatinine, we cannot be sure that the rise in cystatin C actually reflected a true decrease in GFR. To finally answer this question, cystatin C and creatinine must be compared with proper GFR measurements (e.g. inulin or iohexol-clearance) over several ICU days and over a wide range of renal function.

HNL/NGAL

Several studies show that HNL/NGAL is able to predict AKI in a general ICU setting.[139, 152-155] Common to these studies is the over-representation of sepsis among AKI patients. We hypothesized that sepsis will confound the interpretation of plasma HNL/NGAL in AKI since circulating neutrophils release their HNL/NGAL in response to bacterial infections.[91, 92] The origin of urinary HNL/NGAL in AKI is not fully understood. Increased synthesis within the kidney parenchyma has been proposed, but elevated plasma levels may also contribute, and perhaps more so in sepsis, since HNL/NGAL is filtered from the blood.

In Study II we investigated the impact of systemic inflammation and sepsis on the concentrations of HNL/NGAL in plasma and urine. In addition, we assessed the ability of HNL/NGAL to predict AKI in patients with septic shock. We found that plasma HNL/NGAL was elevated in most of our non-AKI patients with SIRS, severe sepsis and septic shock. Furthermore, plasma HNL/NGAL, as well as our markers of systemic

inflammation (CRP), severe bacterial infection (PCT) and neutrophil activation (MPO), gradually increased with sepsis severity. Based on these findings, it appears reasonable to assume that the activated neutrophils represent the main source of the increase in HNL/NGAL levels. However, other organs may also contribute to HNL/NGAL production and secretion into the circulation as a consequence of sepsis and multi-organ damage. Since HNL/NGAL is filtered in the glomeruli, we would expect plasma levels to increase further in septic patients with impaired kidney function. When we compared septic shock patients with and without AKI, however, we found no significant difference in peak levels of HNL/NGAL in plasma. The clinical implication of this was revealed when we found that plasma HNL/NGAL was unable to predict AKI within 12 h in patients with septic shock.

Similarly to HNL/NGAL in plasma, urine concentrations rose in a stepwise manner with increasing sepsis severity. However, the peak concentrations in urine remained below the upper reference limit in most patients without AKI. It could be argued that filtered plasma HNL/NGAL accounted to some extent for the rise in urine. Alternatively, decreased tubular uptake of filtered HNL/NGAL might play a role. Others have suggested that sepsis *per se* triggers albuminuria[156] and that albumin competitively inhibits the megalin receptor-mediated re-uptake of HNL/NGAL and other LMWPs in the proximal tubule.[85] Although we lack information about urinary albumin concentrations, our findings that all three urinary markers (HNL/NGAL, α_1-microglobulin and cystatin C) increased with sepsis severity support this view. Furthermore, it should be noted that three out of seven non-AKI patients with septic shock showed a more than 25% increase in creatinine. These patients may indeed have a mild kidney injury leading to an accumulation of urinary proteins. In contrast to HNL/NGAL in plasma, urinary levels were five-fold higher in AKI patients than in non-AKI patients. Besides, the performance in predicting AKI from urinary HNL/NGAL in septic shock patients was good with an AuROC of 0.86.

It is important to emphasize that a polyclonal antibody-based RIA was used to quantify HNL/NGAL in Study II. Hence, it is likely that all molecular forms of HNL/NGAL (monomeric, dimeric and heterodimeric), if present, were detected in plasma and urine.[157] It was recently shown in an *in vitro* study that kidney epithelial cells mainly

release monomeric HNL/NGAL, whereas neutrophils mainly secrete the dimeric form.[113] Moreover, Cai et al. not only detected both molecular forms in the urine (using Western blot) from patients after cardiac surgery, but also noted that the relative relations between monomeric and dimeric HNL/NGAL varied over time.[114]

The total concentration of urinary HNL/NGAL in AKI, measured by the RIA, probably represents a mixture of different molecular forms of HNL/NGAL with different cellular origins. Urinary monomeric HNL/NGAL levels increase either due to an induced synthesis in the tubular cells or as an effect of impaired reabsorption of the filtered load produced by extra-renal tissues. Furthermore, infiltration of neutrophils in the kidney has been observed in both animal models and in biopsy specimens from patients with AKI.[41, 53] Dimeric HNL/NGAL in urine might emanate from these infiltrating neutrophils, but glomerular filtration of dimeric HNL/NGAL released from activated neutrophils in the circulation, e.g. in septic patients, may also contribute.

In Study IV we used Western blotting to detect monomeric and dimeric HNL/NGAL, respectively, in the urine from critically ill patients. Based on the Western blot results, we examined the ability of two monoclonal ELISAs, with different epitope specificities, to distinguish between the monomeric and dimeric forms.

When we compared the results from the assays with the Western blot patterns (Figure 12) we made some interesting findings. First, the polyclonal RIA measured all three forms of HNL/NGAL as we expected (Figure 12A). Second, we found that the ELISA-1 levels were dependent on the relative amount of monomeric HNL/NGAL. However, ELISA-1 was not monomer-specific since the assay also detected the dimeric form (Figure 12B). Third, ELISA-2 almost exclusively detected dimeric HNL/NGAL. In fact, levels were below the detection limit (< 0.1 μg/l) in 12 out of 20 urine samples with mainly the monomeric form. It must, however, be acknowledged that the absolute ELISA-2 levels were much lower than the ELISA-1 and the RIA levels in samples with mainly the dimeric form (Figure 12C). Hence, it appears that ELISA-2 only detects a fraction of the total amount of dimeric HNL/NGAL. A possible explanation for this might be that some of the specific epitopes which interact with the detecting antibody in ELISA-2 (clone 765) are partially ‘hidden’ when HNL/NGAL is present as a dimer.

Since the kidney epithelial cells mainly release the monomeric form of HNL/NGAL in response to cell damage, it would be of interest to 'remove' the dimeric signal in urine in order to study the state of the tubular epithelial cells. We achieved that by constructing the ELISA-1/ELISA-2 ratio, which amplified the monomeric signal and almost completely distinguished monomeric from dimeric HNL/NGAL (Figure 12D). We therefore concluded that the ELISA-1/ELISA-2 ratio could be used as a more specific measure of tubular epithelial damage than the ELISA-1 alone.

The effect of amplifying the monomeric signal was highlighted when we studied the kinetics of HNL/NGAL, quantified by our two ELISAs, in patients with AKI. A weak but detectable dimeric signal was picked up 24 h prior to the AKI diagnosis and gradually decreased thereafter (Figure 13A). On the other hand, the concentrations measured with ELISA-1 did not change during the observed time frame. This may reflect the fact that ELISA-1 also measured the dimeric HNL/NGAL present in urine during the pre-AKI phase. The monomer-specific ELISA-1/ELISA-2 ratio significantly increased during AKI development (Figure 13B). This increase may represent an induced synthesis of monomeric HNL/NGAL from the tubular cells.

Evidence from animal models suggests that neutrophils mediate tubular injury and play an important role in the development of AKI.[40] It could be speculated that the monomeric and dimeric forms, respectively, reflect different pathophysiological events during the course of AKI. The dimeric signal that we detected prior to the AKI diagnosis might represent an involvement of neutrophils early on in the AKI initiation process. This is supported by several animal studies where depletion or inhibition of neutrophil accumulation in the kidney ameliorated AKI.[158] However, results are conflicting and it is a fact that neutropenic patients are not protected against AKI.

Based on the results in Sudies II and IV, we conclude that plasma HNL/NGAL should be used with caution as a marker of AKI in general ICU patients since sepsis alone increases plasma levels significantly. In contrast, urinary levels are less affected by sepsis and urinary HNL/NGAL is therefore a more robust AKI predictor. In addition, we suggest that, by using a combination of different anti-HNL/NGAL antibodies (ELISA-1/ELISA-2 ratio), it is possible to distinguish between the monomeric and

dimeric forms. This could make the diagnostic accuracy of an HNL/NGAL immunoassay in the urine of patients with suspected AKI much improved since the contribution of neutrophil-derived HNL/NGAL will be, at least partly, eliminated. The ELISA-2 assay may also shed light on the pathogenesis of AKI since the participation of neutrophils is likely in some patients, knowledge which may have a bearing on future treatment strategies.

CONCLUSIONS

- Cystatin C on ICU admission predicts mortality in patients without kidney dysfunction measured by creatinine. Cystatin C predicts long-term mortality independently of AKI and illness severity in the ICU.

- The sepsis-induced inflammatory response does not affect cystatin C levels in plasma.

- HNL/NGAL in plasma is a non-specific predictor of AKI in patients with septic shock since septic shock *per se* increases plasma levels of HNL/NGAL.

- Urinary HNL/NGAL predicts AKI in patients with septic shock.

- A combination of monoclonal antibodies directed against different epitopes on the HNL/NGAL molecule can distinguish between monomeric and dimeric HNL/NGAL in urine.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to all who supported and inspired me during the years, especially:

Claes-Roland Martling, my main supervisor and friend, for breathing life into our ideas and for your razor-sharp thinking and huge amount of common sense. Thank you for believing in me and for being my mentor in everything from renal physiology to personal matters. You are the KING!

Per Venge, co-supervisor and discoverer of HNL, for guiding me into the neutrophil and back again and for teaching me the difference between science and true knowledge. I am forever a fan and indebted.

Max Bell, my dear friend, co-supervisor, and much more, for being a doer, for valuable advice in science and in life and for always encouraging me. Thank you for saving us from the lady with the bat and for being my henchman on the streets of Vicenza.

Shengyuan Xu, co-author, for analysing proteins, especially HNL, with the precision of a Swiss watch. Your contribution to this thesis is invaluable!

Anders Oldner, co-author, for building the robust foundations of clinical research at the Karolinska ICU without which this thesis would never have been possible, for challenging scientific discussions and for being an excellent clinical guide.

Ola Friman, Åsa Bengtsson and Jenny Svedlund, research nurses at the ICU, for your enormous effort in patient recruitment and retrieval of data around-the-clock. This project could not have been carried out without you.

Lena Moberg, Laboratory scientist at the Department of Clinical Chemistry, Uppsala University, for your great effort in visualizing HNL in all its forms.

Kerstin Lindblad and Ann-Katrin Eriksson, Laboratory scientists – deep bows and sincere thanks for skilful technical assistance.

Fredrik Granath, statistical genius, for guiding me through endless STATA outputs.

Matteo Bottai, Professor of Biostatistics at the Department of Environmental Medicine, Karolinska Institutet, for your excellent lectures at Forskarskolan and for helping me with quantile regression.

Anders Larsson, Professor at the Department of Clinical Chemistry, Uppsala, for valuable discussions about cystatin C and its assays, and for your always rapid response when I needed it the most.

Olof Brattström, colleague and fellow student at Forskarskolan, for elaborately describing the trauma cohort.

Gabriella Jäderling, roommate, colleague and fellow PhD student, for speaking the STATA language and for many interesting discussions.

Kirsi Dolk, fourteen thousand cells to fill every 8th week – you impress me! Thanks for excellent scheduling and for providing me with the time to finish my thesis. I needed it, a lot…

Lars Irestedt and **Claes Frostell**, former heads of the Department of Anaesthesia and Intensive Care, for developing the unique spirit at the department, for believing in this project and for giving me the opportunity to combine research and clinical work.

Lars I Eriksson and **Eddie Weitzberg**, Professors at the Department of Anaesthesia and Intensive Care, for sustaining the outstanding research atmosphere at our department. You are consummate scientists and role models!

David Konrad, head of the ICU, for, back then, encouraging me to approach my soon-to-be main supervisor.

Colleagues at the Department of Anaesthesia and Intensive Care, for all your encouragement: it is always a great pleasure to work with you.

The ICU staff, for your commitment to patients and for helping out with sample collections even when the workload is extremely heavy.

Ahrtzing, Lala and Eijlertsson, dear friends, for all the craziness since childhood and reminders of other values than work. **Manne Andersson**, loyal friend, for very pleasant holidays and for the long-distance runs. I look forward to many more adventures together.

My mother and late father, for unconditional love and support. Dad, I'm sorry I didn't finish this book earlier; I know how much you wanted to see it. **Jonas**, my brother, for introducing me to world literature and good music. **Parnee**, my "sister", for being a faithful and caring member of the family

Hucki, my father-in-law, for showing a great interest in my work and for sharing your captivating life stories.

Fanny, for being so generous and wise and for saving us when the wheels are spinning off!

And, finally and most important, my beautiful children, the Fantastic Four: **Nils, Max, Astrid and Filip**, for reminding me of what really matters. I adore you! And **Anja**, my love and best friend. I cannot express with words my gratitude for having you by my side. Your support, encouragement and patience have contributed vastly to the completion of this book.

Generous funding for this thesis was provided by Gambro and Diagnostics Development.

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