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INFLAMMATION AND SEPTIC ACUTE KIDNEY INJURY

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DOCTORAL DISSERTATION

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Unigrafia
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To my family

ABSTRACT

Acute kidney injury (AKI) is a frequent problem in intensive care units (ICUs): during their ICU stay, up to 50% of critically ill adults are diagnosed with AKI. Among them, the most common cause of AKI is sepsis. The risk factors and mechanisms of septic AKI have been updated recently, suggesting fluid balance and inflammation as potential targets for prophylactic and therapeutic interventions. The study objectives were: 1) to investigate the influence of adjusting plasma creatinine for fluid balance on ICU AKI incidence, 2) to study the association of inflammatory and neutrophil activation biomarkers with septic AKI and to assess their temporal variations during early sepsis, and 3) to scrutinize the incremental value of urine neutrophil gelatinase-associated lipocalin (uNGAL) to predict septic AKI and other intensive care outcomes. Studies I–IV utilized clinical data and samples from the national FINNish Acute Kidney Injury (FINNAKI) study. During a 6-month period, all emergency admissions and elective admissions with an expected ICU stay >24 h were included.

Study I included patients who stayed in the ICU for at least 24 h, were not transferred to other ICUs during the first 5 days, were not treated with renal replacement therapy (RRT) before ICU admission, and had recorded weight, fluid input and output data. Daily creatinine (Cr) values from the first five ICU days were adjusted for cumulative fluid balance, and either highest adjusted or highest unadjusted Cr alone or with urine output (UO) and RRT data was applied to obtain AKI incidence, using Kidney Disease: Improving Global Outcomes (KDIGO) criteria. Study I included 2044 patients. The mean difference between adjusted and unadjusted Cr values was 5 (±15) µmol/l. Using adjusted Cr together with UO and RRT (full KDIGO criteria) yielded 19 (1%) additional AKI diagnoses, corresponding to 0.9% (95% confidence interval 0.3–1.6%) absolute difference in AKI incidence. Using only Cr criterium resulted in a slightly larger but not clinically meaningful difference. When the full KDIGO criteria were applied, no 90-day mortality differences existed between the reclassified and those not changing their AKI category.

Study II included a cohort of FINNAKI patients having both 0 h and 24 h plasma samples available and fulfilling severe sepsis criteria, by the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM), between 24 h preceding ICU admission and the second ICU day. Activin A, interleukins 6 (IL-6) and 8 (IL-8), and myeloperoxidase (MPO) were measured from plasma and urine upon ICU admission and from plasma 24 h later, and their associations with AKI were evaluated. Analyzing the 182 patients included in Study II, we found that increased plasma activin A and IL-8 were associated with septic AKI, indicating intravascular neutrophil activation.

Concomitant plasma and urine MPO measurements suggested that urine MPO might not have derived from plasma but possibly reflected renal neutrophil activation associated with septic AKI.

Study III included all Study II patients who had a recorded time label for the first sepsis-associated organ dysfunction (OD). To assess the kinetics of heparin binding protein (HBP), MPO, IL-6, and IL-8 in early sepsis, we compared their concentrations between healthy controls and three patient groups: those having the plasma sample taken >1 h before OD emergence, those with sample taken close to the first OD emergence (±1 h), and those with sample taken >1 h after OD emergence. Study III included 167 sepsis patients. Healthy controls had lower plasma HBP, MPO, IL-6, and IL-8 levels than sepsis patients. Unlike HBP and MPO, IL-6 and IL-8 levels differed among sepsis patients according to the sample timing: highest values were observed among those with sample obtained >1 h after the first OD and lowest among those with sample taken >1 h before the first OD.

Study IV included all sepsis patients meeting the latest Sepsis-3 criteria from the previous FINNAKI-NGAL study. To evaluate the clinical applicability of uNGAL, we first constructed clinical risk models using the routinely available admission data without uNGAL. We then assessed the ability of uNGAL to improve AKI, RRT, and 90-day mortality prediction in sepsis patients using recently introduced statistical instruments: risk assessment plot (RAP) and decision curve analysis (DCA). Analyzing the 484 sepsis patients included in Study IV, we found that adding uNGAL to the clinical risk models led to modest improvement in AKI and RRT prediction, but no change in 90-day mortality prediction. With test trade-offs between 40 (for AKI) and 74 (for RRT), we cannot recommend uNGAL for clinical use in this patient group.

To conclude, adjusting Cr for fluid balance was unnecessary to diagnose and stage AKI or to detect high–risk ICU patients if the full KDIGO criteria, including UO, were applied. Septic AKI and systemic neutrophil activation were linked. Concomitant plasma and urine neutrophil activation marker measurements suggested neutrophil accumulation in the kidneys of patients with septic AKI. Systemic elevation of neutrophil activation markers HBP and MPO was an early event in sepsis, appearing before the first OD emerged. HBP and MPO plasma levels were not influenced by the sampling time within the first 24 h following ICU admission, unlike IL-6 and IL-8. uNGAL, a proposed biomarker for AKI prediction among sepsis patients, did not offer clinically meaningful incremental value to predict AKI or other relevant clinical outcomes.

Keywords: acute kidney injury, fluid balance, creatinine, incidence, mortality, activin A, interleukin 6, interleukin 8, myeloperoxidase, sepsis, critical illness, intensive care, heparin binding protein, neutrophil gelatinase-associated lipocalin

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Helsinki, January 2023

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II Törnblom S, Nisula S, Vaara ST, Poukkanen M, Andersson S, Pettilä V, Pesonen E. Neutrophil activation in septic acute kidney injury: A post hoc analysis of the FINNAKI study. Acta Anaesthesiol Scand 2019; 63: 1390–1397.

III Törnblom S, Nisula S, Vaara ST, Poukkanen M, Andersson S, Pettilä V, Pesonen E. Early prolonged neutrophil activation in critically ill patients with sepsis. Innate Immunity 2021; 27(2): 192–200.

IV Törnblom S, Nisula S, Petäjä L, Vaara ST, Haapio M, Pesonen E, Pettilä V, and the FINNAKI study group. Urine NGAL as a biomarker for septic AKI: a critical appraisal of clinical utility—data from the observational FINNAKI study. Annals of Intensive Care. 2020; 10(1): 51.

The publications are referred to in the text by their Roman numerals.

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ABBREVIATIONS

ACCP	American College of Chest Physicians
AKI	acute kidney injury
AKIN	Acute Kidney Injury Network
APACHE II	acute physiology and chronic health evaluation II
ATHOS-3	Angiotensin II for the Treatment of Vasodilatory Shock 3
ATP	adenosine triphosphate
AUROC	area under the receiver operating characteristic (curve)
cfNRI	category-free net reclassification improvement
CI	confidence interval
CKD	chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
COVID-19	coronavirus disease 2019
CrCl	creatinine clearance
CRF	case report form
CRP	c-reactive protein
DAMP	damage-associated molecular pattern
DCA	decision curve analysis
ELISA	enzyme-linked immunosorbent assay
FINNAKI	FINNish Acute Kidney Injury (study)
GFR	glomerular filtration rate
HBP	heparin binding protein
ICU	intensive care unit
IDI	integrated discrimination improvement
IGFBP-7	insulin-like growth factor-binding protein 7
IL	interleukin
IQR	interquartile range
KDIGO	Kidney Disease: Improving Global Outcomes
KIM-1	kidney injury molecule 1
LPS	lipopolysaccharide
MAP	mean arterial pressure
MDRD	Modification of Diet in Renal Disease Study Group
MPO	myeloperoxidase
OD	organ dysfunction
PAMP	pathogen-associated molecular pattern
RAP	risk assessment plot
RBF	renal blood flow
RIFLE	Risk, Injury, Failure, Loss, End-stage

RRT	renal replacement therapy
SAPS II	simplified acute physiology score II
SCCM	Society of Critical Care Medicine
(s)Cr	(serum) creatinine
SIRS	systemic inflammatory response syndrome
SOFA	sequential organ failure assessment
sTREM-1	soluble triggering receptor expressed on myeloid cells 1
TIMP-2	tissue inhibitor of metalloproteinases 2
TLR	toll-like receptor
TNF(α)	tumor necrosis factor (alpha)
(u)NGAL	(urine) neutrophil gelatinase-associated lipocalin
UO	urine output

1 INTRODUCTION

Acute kidney injury (AKI) is a common complication among acutely hospitalized adult patients: it affects approximately one in five of them [1]. The latest definition and staging of AKI, by the Kidney Disease: Improving Global Outcomes (KDIGO) workgroup, is based on creatinine (Cr), urine output (UO) and administered renal replacement therapy (RRT). AKI incidence and the need for RRT are growing as the population ages [2,3]. Currently, AKI incidence among critically ill patients is reported to be 32–50% [4,5], of which roughly 50% is caused by sepsis [6]. Beyond acute morbidity, AKI contributes to long-term illness and mortality since it predisposes individuals to chronic kidney disease and associated health problems [7]. After an AKI episode, deterioration of kidney function is more likely to happen—a phenomenon called "accelerated ageing"—despite normalized Cr values following the primary insult [8].

Earlier interpretation of the pathophysiology of sepsis-induced AKI, or septic AKI for short, emphasized macrocirculatory mechanisms [9]. Based on experimental studies, it was suggested that lowered mean arterial pressure (MAP) and vasoconstriction would result in diminished renal blood flow (RBF), renal ischemia, tubular necrosis, and the clinical AKI syndrome. Recently, this macrocirculatory theory as the primary mechanism of septic AKI has been questioned. First, post-mortem studies of sepsis patients' kidneys have not verified significant tubular necrosis [10] or even apoptosis [11]. Second, a critically ill patient frequently develops AKI even though RBF is normal or elevated [12]. In fact, a large emergency department study revealed an increased immune response and AKI in every fourth hemodynamically stable pneumonia patient not even needing intensive care [13]. Experimental evidence of inflammatory mechanisms in septic AKI is growing as well [14,15].

Based on the new evidence, Gomez and colleagues [16] have introduced a unified theory of septic AKI. According to the theory, pathophysiological mechanisms of septic AKI include activated inflammatory cascades, disordered and heterogenous renal microvascular flow, and tubular epithelial cell impairment [16]. The inflammatory response to pathogen invasion is triggered by pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS), interacting with pattern recognition receptors. These in turn activate leukocytes and complement and coagulation pathways. Necrotic cell death releases damage-associated molecular patterns (DAMPs) that also bond to pattern recognition receptors, further intensifying inflammation [17]. Early sepsis mortality is caused by an inappropriately intense systemic inflammatory

response, characterized by a "cytokine storm", the following widespread organ dysfunction (OD), and, eventually, irreversible multiple organ failure.

The kidney meets the inflammatory burst in the frontline since it receives 20% of cardiac output and filtrates 120–150 milliliters of plasma each minute. Indeed, AKI, manifested by oliguria, is often among the first emerging ODs after sepsis onset. The microcirculatory dysfunction observed in sepsis slows down the peritubular blood flow and increases the tubular cells' exposure time to activated leukocytes, PAMPs, DAMPs, and cytokines mediating inflammation. The danger signal is passed on to more distal tubular cells by paracrine signaling. This leads to cell metabolism reprioritization, cell cycle arrest, and shutdown of kidney function, observed as clinical AKI [16].

Clinical evidence on the association of inflammation with septic AKI is grounded on only a few interleukin studies. In the Prospective Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis trial placebo group, high interleukin 6 (IL-6) plasma concentration, not macrocirculatory parameters, was associated with septic AKI [18]. A similar association between IL-6 and AKI was observed among pneumonia patients [13]. Payen and colleagues [19] found that IL-6 and IL-10 correlated with AKI severity, being highest in the most severe AKI. The interleukin concentrations decreased rapidly during the first three intensive care unit (ICU) days [19]. IL-6 has also shown association with sepsis severity [13].

Neutrophil activation is the key point in the early innate immune response, and, according to Gomez and colleagues [16], septic AKI development. Therefore, measuring neutrophil activation markers is clinically tempting. Activin A, stored in the neutrophil cytosol [20-22], is rapidly released into the plasma from activated neutrophils—elevating even earlier than IL-6 in experimental sepsis [23,24] and correlating with disease severity and mortality in sepsis patients [25]. Heparin binding protein (HBP) and myeloperoxidase (MPO) are neutrophil granule markers, of which HBP is released mostly intravascularly and MPO upon neutrophil activation in the tissues [26]. Among sepsis patients, plasma HBP predicts AKI [27,28] and OD in general [29]. Plasma MPO is associated with the development of OD and mortality [30,31].

AKI biomarkers have been under intense research over the years. Neutrophil gelatinase-associated lipocalin (NGAL) has shown promise in predicting AKI after cardiac surgery, but less success with regards to heterogenous intensive care patients [32]. Proinflammatory cytokines, such as IL-18 [33], and leukocyte surface receptors, such as soluble triggering receptor expressed on myeloid cells 1 (sTREM-1) [34], have also not performed well enough in clinical studies. The sensitivity and specificity of new cell cycle markers, for instance, tissue inhibitor of metalloproteinases 2 (TIMP-2) and insulin-like growth factor-binding protein 7 (IGFBP-7) might be better [35], and combining several markers

could be useful. Nonetheless, a truly kidney-specific marker, "renal troponin", is yet to be found.

Despite experimental evidence and evolving theories, we still do not have proper tools to predict, prevent, or treat septic AKI. According to the latest knowledge, the kidneys adapt to the overwhelming inflammatory burst by shutting down all non-essential functions early in the course of septic infection. Supporting this, Murugan and colleagues showed that almost two-thirds of hospitalized adult pneumonia patients who developed AKI already fulfilled the criteria when arriving at the emergency room [13]. Even so, AKI diagnosis relies on a late functional marker, Cr, which possibly dilutes following fluid resuscitation, further postponing AKI diagnosis. Under these circumstances, carrying out timely preventive interventions is challenging, to say the least.

Although experimental evidence is growing, our knowledge on the human inflammatory reaction and septic AKI pathophysiology is scarce. Considering the presented concern about the effect of fluid accumulation on ICU AKI diagnoses, we first studied the influence of cumulative fluid balance on the KDIGO AKI diagnosis to find out whether Cr dilution affects ICU AKI incidence. The main aims of the study were to investigate the association of inflammation and neutrophil activation with septic AKI, to address the kinetics of proinflammatory cytokines and neutrophil activation markers in sepsis patients around the OD onset, and to test if urine NGAL (uNGAL) is useful to predict AKI in critically ill sepsis patients.

2 REVIEW OF THE LITERATURE

2.1 Defining, staging, and diagnosing septic AKI

2.1.1 Sepsis definition

Sepsis, a phenomenon recognized in antiquity, has sowed horror through the centuries and is still a considerable burden to intensive care globally [36]. Reflecting the complexity of the syndrome, the first definition (Sepsis-1) was not published until 1992 [37]. This consensus statement by the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) was based on the concept of systemic inflammatory response syndrome (SIRS), describing the physiological derangements caused by the host's response to infection. SIRS was considered present when a patient had more than one of the following clinical findings: body temperature higher than 38°C or lower than 36°C, heart rate higher than 90 beats/min, hyperventilation evidenced by respiratory rate higher than 20 breaths/min or partial pressure of carbon dioxide lower than 4.3 kPa in arterial blood, and white blood cell count higher than 12.0 E9/l or lower than 4.0 E9/l, or >10% immature neutrophils. Sepsis was defined as SIRS due to a proven or suspected infection. If acute OD, defined, for example, by Sequential Organ Failure Assessment (SOFA) score [38] (Table 1), was simultaneously present, criteria for severe sepsis were met. Septic shock, the most serious sepsis manifestation, was defined as sepsis with persisting hypotension, or need for vasopressors, despite adequate fluid resuscitation and unexplained by other causes. The Sepsis-2 definition from 2001 added weight on the clinical judgement of an infectious etiology and introduced new inflammatory and OD parameters and SIRS components. The main concept stayed the same though [39].

The inadequate specificity and sensitivity of SIRS criteria attracted criticism. It is well known that SIRS frequently exists without infection: for instance, with pancreatitis, sterile burns, after major surgery. Kaukonen and colleagues showed that the need for two or more SIRS criteria to define severe sepsis excluded one in eight otherwise similar patients with infection, organ failure, and substantial mortality, and failed to define a transition point in the risk of death [40]. Accordingly, the latest sepsis definition from 2016, Sepsis-3, omitted SIRS and the term severe sepsis [41]. The Sepsis-3 definition also abandoned the idea of a continuum through severe sepsis to septic shock. The new definition describes sepsis as life-threatening OD caused by a dysregulated host response to infection. Based on the finding that general hospital patients

with presumed infection and ≥2 SOFA OD points already have a notable 10% overall mortality risk [42], the revised sepsis definition requires an infection-related acute increase of more than two points from the patient's baseline SOFA score [38] (Table 1). The updated septic shock definition stipulates infection associated with persistent hypotension requiring vasopressors to maintain adequate tissue perfusion and a blood lactate >2 mmol/l despite adequate fluid resuscitation. The former (Sepsis-1) and the current (Sepsis-3) definitions are compared in Table 2. Outside the ICU, the quick SOFA score (Table 3) is one recommended tool to differentiate potential sepsis cases among patients with infection [41].

Table 1 Sequential Organ Failure Assessment (SOFA) Score

	CNS	Cardiovascular system	Respiratory system	Coagulation	Liver	Renal function
Score	**GCS**	**MAP OR administration of vasopressors required**	**PaO_2/FiO_2, mmHg**	**Platelets, $\times 10^3/\mu l$**	**Bilirubin, µmol/L**	**Cr, µmol/L (or UO)**
+0	15	MAP ≥70 mmHg	≥400	≥150	<20	<110
+1	13–14	MAP <70 mmHg	<400	<150	20–32	110–170
+2	10–12	dopamine ≤5 µg/kg/min OR dobutamine (any dose)	<300	<100	33–101	171–299
+3	6–9	dopamine >5 µg/kg/min OR epinephrine ≤0.1 µg/kg/min OR norepinephrine ≤0.1 µg/kg/min	<200 AND mechanically ventilated (including CPAP)	<50	102–204	300–440 (OR <500 ml/d)
+4	<6	dopamine >15 µg/kg/min OR epinephrine >0.1 µg/kg/min OR norepinephrine >0.1 µg/kg/min	<100 AND mechanically ventilated (including CPAP)	<20	>204	> 440 (OR <200 ml/d)

CNS, central nervous system; CPAP, continuous positive airway pressure; Cr, creatinine; GCS, Glasgow Coma Scale; MAP, mean arterial pressure; PaO2/FiO2, ratio of the partial pressure of oxygen in arterial blood to the inspired oxygen fraction; UO, urine output

Table 2 Comparison of Sepsis-1 and Sepsis-3 criteria.

	Sepsis-1 (1992)	Sepsis-3 (2016)
Sepsis	Known/suspected infection + ≥2/4 SIRS criteria: temperature >38°C or <36°C, heart rate >90/min, respiratory rate >20/min or pCO_2 <4.3 kPa or mechanical ventilation, leukocyte count >12E9/l or <4 E9/l or >10% immature neutrophils	Known/ suspected infection + acute increase of SOFA ≥2
Severe sepsis	Sepsis + organ dysfunction	Not a category
Septic shock	Sepsis + refractory hypotension/vasopressor need after adequate fluid resuscitation	Sepsis + vasopressors and lactate >2 mmol/l

SIRS, systemic inflammatory response syndrome; SOFA, sequential organ failure assessment

Table 3 The Quick SOFA criteria.

Respiratory rate ≥22 breaths/min
Systolic blood pressure ≤100 mmHg
Altered mentation (GCS <15)

GCS, Glasgow Coma Scale

Like SIRS, SOFA has its shortcomings from the clinician's perspective. As an example, to define cardiovascular SOFA points, the chosen vasopressor/ inotrope medications and their cut-off doses can be questioned, especially when treating sedated patients using fluid-restrictive strategies. These patients easily get high cardiovascular SOFA points that do not necessarily reflect true disease severity, especially when compared with other organ system points. Another obvious problem is that renal SOFA points do not take ongoing RRT into account.

2.1.2 AKI definition and staging

AKI is a clinical syndrome of sudden kidney function decline, manifested by a decrease in glomerular filtration rate (GFR). The first AKI definition (RIFLE: Risk, Injury, Failure, Loss, End-stage)—based on serum creatinine (sCr), estimated GFR, and UO—was proposed in 2004 [43]. With some changes made by the Acute Kidney Injury Network (AKIN), the AKIN criteria for AKI were published a few years later [44]. The latest guidelines for diagnosing and staging AKI were released by the KDIGO workgroup in 2012 [45]. They harmonized pediatric and adult criteria and included both relative and absolute sCr changes.

Using the latest KDIGO definition, an individual has AKI when any of the following three criteria are present:

- sCr increase ≥0.3 mg/dl (≥26.5 µmol/l) within 48 h, or
- sCr increase to ≥1.5 times baseline, presumed or known to have occurred in the previous 7 days, or
- UO <0.5 ml/kg/h for 6 h.

All three (RIFLE, AKIN, and KDIGO) lean on GFR surrogates sCr and UO, and stage AKI into categories of increasing severity. The characteristics and differences between these three definitions are illustrated in Table 4.

Table 4 Comparison of RIFLE, AKIN and KDIGO criteria for AKI diagnosis and staging.

	RIFLE criteria		AKIN criteria	KDIGO criteria	All
R(isk)	sCr ≥1.5 x baseline within 7 days for ≥24 h or >25% decrease in eGFR	**Stage 1**	sCr ≥1.5 x baseline or increase of ≥26.2 µmol/l within 48 h	sCr ≥1.5 x baseline within 7 days or ≥26.2 µmol/l increase within 48 h	Diuresis <0.5 ml/kg/h for 6–12 h
I(njury)	sCr ≥2.0 x baseline or >50% decrease in eGFR	**Stage 2**	sCr ≥2.0 x baseline	sCr ≥2.0 x baseline	Diuresis <0.5 ml/kg/h for ≥12 h
F(ailure)	sCr ≥3.0 x baseline or >75% decrease in eGFR or an absolute sCr ≥354 µmol/l with an acute rise of at least 44 µmol/l	**Stage 3**	sCr ≥3.0 x baseline or an absolute sCr ≥354 µmol/l with an acute rise of at least 44 µmol/l or initiation of RRT	sCr ≥3.0 x baseline or an absolute sCr ≥354 µmol/l or initiation of RRT	Diuresis <0.3 ml/kg/h for ≥24 h or anuria for ≥12 h

AKI, acute kidney injury; AKIN, Acute Kidney Injury Network; eGFR, estimated glomerular filtration rate; KDIGO, Kidney Disease: Improving Global Outcomes; RIFLE, Risk, Injury, Failure, Loss, End-stage; RRT, renal replacement therapy; sCr, serum creatinine

2.1.3 Measuring kidney function

The best overall measure of kidney function is renal filtering capacity, GFR. It is a measure of the amount of plasma-like fluid filtered through the glomerular capillaries into the renal tubules in a time unit. A young healthy adult's GFR is 120–130 ml/min/1.73m^2, but it declines with age and >90 ml/min/1.73m^2 is generally considered normal. GFR is calculated using the following equation:

$$GFR\ (ml/min) = (Cu \times Qu)/Cp$$

Cu = concentration of a substance in urine (mg/ml)
Cp = concentration of a substance in arterial plasma (mg/ml)
Qu = urine flow rate

Ideally, the substance used to measure GFR must be freely filtered through glomeruli—not secreted nor reabsorbed by tubular cells. Inulin clearance is the "gold standard", but exogenous substances are not feasible in everyday clinical practice. Instead, creatinine clearance (CrCl) is used as a surrogate, although 5% of Cr is excreted in the proximal tubule and the excretion rate depends on sCr concentration [46]. Thus, CrCl overestimates GFR by 10–20%. Easily obtainable serum (or plasma) Cr, a breakdown product of dietary meat and creatine phosphate of skeletal muscle, is dependent on an individual's age, sex and race, his/her muscle mass, catabolic rate, and fluid status [46-48]. It is also well known that certain medications may influence sCr levels. An alternative biomarker for GFR estimation is cystatin C, a basic endogenous protein produced by all nucleated cells and thereby not dependent on a person's muscle mass [49]. Dietary protein intake does not influence cystatin C [50]. It is freely filtered at the glomeruli and almost completely reabsorbed and metabolized in the proximal tubule [49]. Cystatin C is particularly useful to estimate GFR in early renal insufficiency when Cr is still within normal limits [51].

KDIGO AKI definition involves a sCr increase from the baseline. Without recent Cr measurement, the baseline kidney function of an acutely ill patient is unknown—Cr could have been elevated chronically. Under steady state conditions, GFR (or CrCl) for a patient without chronic kidney disease (CKD) can be estimated using one of several existing equations that include sCr and other variables influencing GFR. Unfortunately, neither Cr production nor elimination are stable in evolving AKI, meaning that none of the existing equations are really accurate in these patients.

The Modification of Diet in Renal Disease Study Group (MDRD) formula, usually the simplified 4-variable modification, has been widely used for adults, and was the recommended equation during our study enrollment period [52].

The MDRD equation (simplified 4-variable equation without urea and albumin, International System of Units):

$$GFR\ (ml/min/1.73m^2) = 175 \times (sCr/88.4)^{-1.154} \times age^{-0.203} \times (0.742\ \text{if female}) \times (1.212\ \text{if of African descent})$$

Recently, the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula has become the recommended equation for clinical practice [53,54]. With CKD-EPI, either Cr or cystatin C, or both, can be used to calculate GFR [55].

2.1.4 Diagnosis of septic AKI

Diagnosing AKI requires clinical judgement. Although ancillary tests and checklists may help in the process [56], the KDIGO definition itself can be troublesome. First, the observation periods determining anuria/oliguria in the current KDIGO definition and staging are somewhat arbitrary: a patient with oliguria for 5 h 59 min hardly "develops AKI" a minute later. Second, a critically ill or deteriorating patient, admitted to ICU from the emergency department or from a hospital ward, often comes without preceding UO data. Then, Cr becomes the sole applied KDIGO criterion for AKI diagnosis. This can be problematic because high-volume fluid resuscitation administered to a critically ill patient may dilute sCr, and the dilution effect may mask or delay AKI diagnosis [47,57,58]. Therefore, adjusting Cr for fluid balance has been suggested [59], but the rationale for the available adjustment equations has been criticized for using several days' cumulative fluid balance to correct a single Cr measurement [60].

Third, a fundamental weakness in the present AKI definition is that neither sCr elevation nor UO decrease are early signs of injury. There is "renal reserve": deteriorating kidney function is not manifested by Cr elevation until half the kidney function has already been lost. Furthermore, when a steady state has not been achieved as in evolving AKI, sCr poorly reflects GFR. Traditionally recommended urine biochemistry (fractional excretion of sodium and urea) does not offer any beneficial information for differential diagnosis or AKI prognosis in the ICU [61] and has been abandoned in many countries, including Finland. To promote more rapid diagnosis, several novel kidney injury biomarkers have been studied [62,63]. Some may be included in the future AKI definitions and staging [64].

As stated previously, by the current Sepsis-3 definition, sepsis is accompanied by acute OD. If OD emerges in the kidneys and meets the KDIGO AKI criteria, septic AKI is present. Because diagnosing each of these two syndromes—sepsis and AKI—requires clinical judgement, diagnosing septic AKI is not always straightforward. A high level of vigilance is warranted to identify these high-risk patients.

2.2 Epidemiology of septic AKI

Sepsis is the most common causative factor for AKI among ICU patients [65]. A study from Australia and New Zealand estimated the annual population incidence of ICU-treated sepsis to be 0.77/1000 adults per year [66]. Sepsis incidence in the western world is rising along with the aging population's increasing prevalence of chronic diseases, invasive medical procedures

and disease- and therapy-related immunosuppression, as well as antibiotic resistance [67]. Cases of septic AKI will probably increase accordingly. In Finland, our national prospective cohorts have showed an increase in the incidence of ICU-treated severe sepsis from 0.39/1000 adults per year (2005) to 0.60/1000 adults per year (2012) [68,69]. Sepsis incidence has been studied predominantly in ICUs with convenient routinely collected data, perhaps because patients outside ICUs do not have that much data on sepsis and infection diagnoses. Obviously, the entire burden of sepsis is much broader than the tip of the iceberg observed within intensive care in high-income countries. In the United States, for example, half the patients with severe sepsis are treated outside ICUs [70]. Many of these patients may develop septic AKI [13,70]. Moreover, a recent worldwide study of hospital and death records revealed much higher sepsis incidence in low-income countries than in the United States or Europe [71], but no prospective studies have been conducted to unravel sepsis epidemiology globally [72]. The effect of the new Sepsis-3 definition on incidence is unknown.

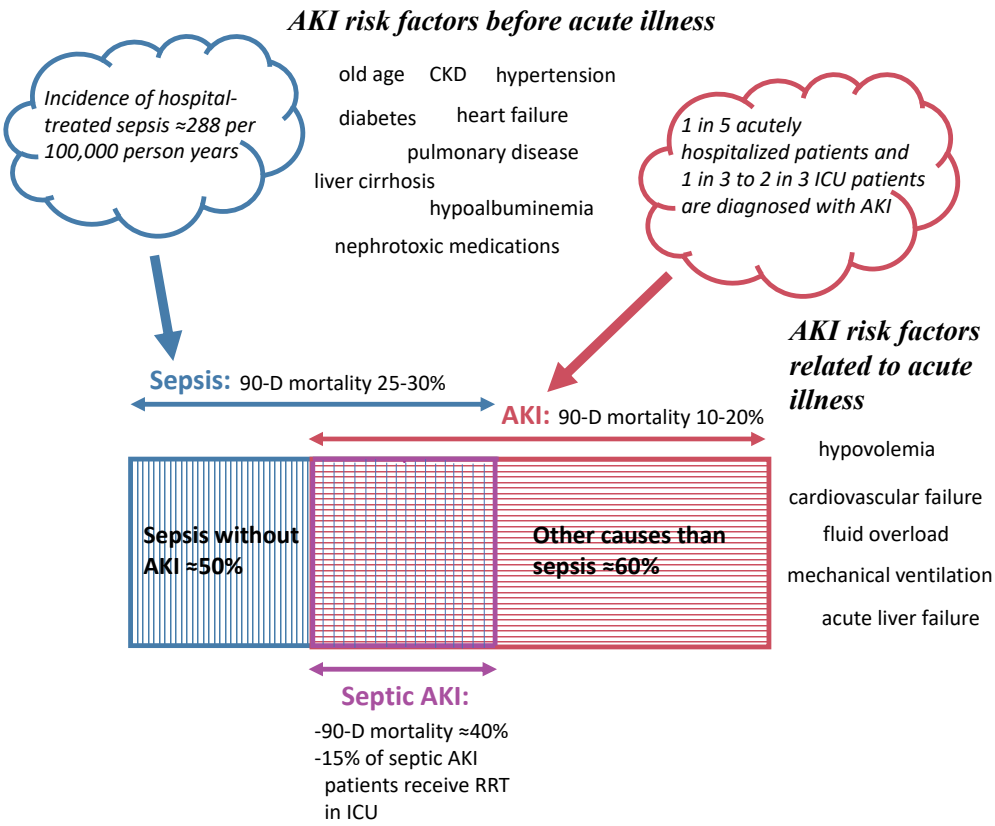


Figure 1 AKI in ICU patients.

AKI, acute kidney injury; CKD, chronic kidney disease; D, day; ICU, intensive care unit; RRT, renal replacement therapy. Figure by the author.

Previously, varying AKI definitions made it impossible to determine septic AKI incidence precisely. This age of unawareness continued until the beginning of the 21st century, when generally accepted AKI definitions started emerging. Recent studies have reported that 45–53% of ICU sepsis patients are diagnosed with AKI [69,73]. Well-known risk factors for all-cause AKI include CKD, advanced age, chronic or acute cardiovascular failure, diabetes, chronic or acute liver disease, hypoalbuminemia, pulmonary disease, and mechanical ventilation [74]. There are no commonly established validated risk scores for septic AKI. Figure 1 shows the crude AKI prevalence in hospitalized patients and contributing factors among the critically ill.

2.3 Genetics of septic AKI

Genetic susceptibility to AKI has aroused research interest recently. Although two unrelated persons share 99.9% of their DNA sequences, there are variations known as gene polymorphisms, resulting in differing gene variants, or alleles. These variations producing biological diversity can range from a single nucleotide polymorphism to a 10–200 nucleotide sequence, repeated 5–50 times between two restriction sites and characterized by many alleles. Some of the polymorphisms cause production of an abnormal protein and some correlate with specific phenotypes relevant to human disease. These polymorphisms are not necessarily incriminated in the disease pathogenesis but may merely be located in proximity to other pathogenic genetic factors, which is called "linkage disequilibrium".

Some evidence of genetic susceptibility to septic AKI exists, according to studies on genes that encode inflammatory mediators. Although the overall quality of these genetic studies is not that good [75], some examples are worth mentioning. Polymorphism of the gene encoding tumor necrosis factor alpha (TNFα) influences TNFα concentration, and a high concentration is associated with multiple organ dysfunction syndrome [76]. Vascular endothelial growth factor is a chemoattractant, augments macrophage differentiation, activates platelets and increases vascular permeability, and the hypersecretory phenotype is associated with AKI [77]. MPO, a phagolysosomal enzyme and neutrophil activation marker, has lower plasma concentrations in some MPO-gene haplotypes that are associated with a reduced need for RRT [78]. Gene polymorphisms of proinflammatory cytokines IL-6 and IL-8, on the other hand, have not been associated with AKI among general intensive care patients or ICU patients with severe sepsis [77,79]. Most of the reported associations have not been reproducible. It is thus likely that single polymorphisms have little impact and predisposition to AKI is polygenic.

Reflecting the polygenic nature of septic AKI, recent studies in sepsis patients have identified genetically and clinically distinct endotypes that react differently to bacteremia regarding inflammatory response [80-82]. Using routinely available clinical data on hospital presentation, Seymour and colleagues identified four sepsis phenotypes, reproducible across different datasets [81]. These phenotypes differed in their inflammatory response, coagulation derangement and kidney injury markers, and mortality. Phenotypes γ and δ with the highest levels of inflammatory cytokines IL-6, IL-10 and TNF were admitted to intensive care more frequently and had higher in-hospital mortality than the less inflammatory phenotypes α and β. The phenotypes were not distinguishable by infection site, illness severity, or OD patterns alone [81]. The heterogenous responses of the distinct phenotypes might explain the modest overall treatment effects observed in immunomodulatory trials of unselected sepsis populations.

2.4 Pathophysiology of septic AKI

2.4.1 Histopathology

Based on experimental findings of ischemia-reperfusion models, acute tubular necrosis was also thought to be the key histopathological feature in septic AKI. Current evidence indicates, however, that ischemic and septic AKI are profoundly different [10,83]. Contradicting the earlier hypothesis of acute tubular necrosis, recent experiments in sheep with septic AKI detected practically no renal morphological changes at all, even though researchers observed a substantial increase in Cr and decrease in GFR and UO [84,85]. A similar paucity of morphological changes has been reported in human postmortem studies as well. Only minor focal tubular lesions, leukocyte infiltration and scarce apoptosis have been found in septic kidneys, but most nephrons look normal, and the histological changes are not consistent with the functional deficiency [10,11].

2.4.2 Macrocirculation and renal blood flow (RBF)

The former theory of septic AKI was constituted on the grounds of experimental endotoxin studies. It was assumed that diminished RBF and ischemia following systemic vasodilation and hypotension caused the clinical septic AKI syndrome [9]. Attempts to treat septic AKI were therefore targeted to maintain RBF by increasing cardiac output and perfusion pressure—first and foremost using intravenous fluids. Since then, growing evidence has challenged overly aggressive fluid loading and macrocirculatory targets aiming at “normal”

or even higher than that. As an example, in the SEPSISPAM study of septic shock patients undergoing resuscitation, a MAP target of 80 to 85 mmHg, as compared with 65 to 70 mmHg, did not result in significant differences in RRT demand or in the endpoint of plasma Cr doubling [86]. Additionally, successfully resuscitated out-of-hospital cardiac arrest patients with prolonged warm ischemia of the kidneys seldom develop AKI [87]. Further evidence is presented by a study comprising 159 original articles that use animal models. This study reported that RBF findings in experimental sepsis depend on the model used, but in sepsis with increased cardiac output, RBF is typically increased [88]. Decreased RBF is not a universal finding in human septic or non-septic AKI either, according to a review by Prowle and colleagues [12], but positive fluid balance is associated with AKI and other adverse outcomes [89-91]. As a matter of fact, a pilot study of 100 ICU patients indicates that restrictive fluid management may reduce the need for RRT [92]. Aiming at traditional resuscitation targets, such as certain mean arterial or central venous pressure, may cause more harm than benefit for the kidneys.

2.4.3 Microcirculation and endothelial dysfunction

How, in the situation of normal or even increased RBF, is GFR decreased, then? First, greater efferent than afferent glomerular dilatation lowers intraglomerular filtration pressure in sepsis [93-96]. Second, endothelial dysfunction and glycocalyx damage increase vascular permeability. The resulting tissue edema and congestion may raise venous output pressures inside the renal capsule, further challenging kidney function. Third, renal microcirculation in peritubular and glomerular areas, not the global RBF, may be the key player—particularly because it lies between tubular endothelium and circulating immune cells [97]. Indeed, in renal microcirculation of animals with experimental sepsis, there are areas of sluggish peritubular flow [98]. Furthermore, evidence exists of intercellular adhesion molecule 1 and vascular cell adhesion molecule 1 being up-regulated in the peritubular endothelium, which contributes to prolonged leukocyte transit and increased signaling with kidney dendritic cells [99]. An LPS model of murine sepsis demonstrated that tubular cell redox stress was highly correlated with the percentage of dysfunctional capillaries [99]. Gomez and colleagues suggest that the prolonged transit of activated leukocytes through dysfunctioning capillaries exposes tubular epithelial cells to excessive proinflammatory cytokine load and to abundant PAMPs and DAMPs that amplify the inflammatory signal and cause oxidative stress [16]. Corroborating this, incubating cultured human tubular cells in the plasma of septic burn patients with AKI induces cell damage and dysfunction [100].

2.4.4 Role of neutrophils

During the early hours and days of infection, our defense against a newly encountered microbe relies on rapid recognition of the intruder as "non-self" and the following innate immune system activation programed to destroy the pathogens before infection spreads. Comprehensive experimental data confirms that neutrophils, polymorphonuclear phagocytic cells, are essential participants in the early innate immune response to infection. Under normal conditions, most neutrophils are stored in the bone marrow. Only a small circulating fraction can be observed, and healthy tissues are essentially neutrophil-free [101]. Intruding bacteria and their specific molecular features (PAMPs) activate pattern recognition receptors on immune, endothelial and epithelial cells. This triggers proinflammatory transcription factors that increase the transcription of genes encoding mediators and receptors necessary for systemic inflammation [102]. Chemotactic mediators, such as IL-8, produced by macrophages, epithelial and endothelial cells, recruit neutrophils to the infection site [103,104]. Neutrophils express toll-like receptors (TLRs) themselves and react to PAMPs and DAMPs by secreting inflammatory mediators and chemokines that further amplify inflammation and lure more and more neutrophils from their bone marrow storage [105]. Inflammatory mediators activate not only neutrophils, but also thrombocytes and the coagulation pathway.

Activated neutrophils are short-lived [106] and destined to die killing bacteria [107]. From their cytosol and intracellular granules, activated neutrophils sequentially release activin A (a cytokine promoting neutrophil phagocytic function and oxidative burst) [21,108], HBP (a protein with multiple functions including regulation of vascular permeability), and MPO (an enzyme producing cytotoxic agents) [26,109]. Excessive systemic toll-like receptor 2 (TLR2) stimulation can lead to increased cell death and exhaustion of the bone marrow neutrophil reservoir resulting in neutropenia and a worse prognosis [110]. Although TLR2-mediated neutrophil activation is essential to limit infection at the site, overly extensive systemic TLR2 signaling also causes downregulation of C-X-C motif chemokine receptor 2 surface expression and impaired neutrophil migration to the infection site [102]. At the same time, activated neutrophils upregulate their surface expression of C-C chemokine receptor type 2, a receptor undetectable on "resting" neutrophils, enhancing neutrophil migration into tissues other than the infection focus [102]. Subsequent neutrophil infiltration and cytokine release contribute to damage and secondary OD in distant organs [111,112]. Septic AKI experiments in animals have shown that neutrophils infiltrate the kidney and that the amount of infiltration correlates with the degree of renal dysfunction. Correspondingly, neutrophil depletion and other interventions reducing renal neutrophil accumulation have a protective effect [14,15,113-116]. Since endothelial cells also express TLRs and become activated, resulting adhesion of activated

circulating neutrophils and thrombocytes to capillary walls causes occlusion of flow and tissue ischemia (Figure 2).

2.4.5 Cellular response to danger and kidney function in sepsis

As stated above, it is not necrosis or large-scale apoptosis that causes kidney dysfunction during sepsis. Instead, a torrent of mediators reaches the kidney, altering its normal functions. Initially, circulating PAMPs and DAMPs are recognized by TLR2- and TLR4-expressing epithelial cells of the proximal tubules. From those alarmed proximal tubular cells, the danger signal is conveyed to the adjacent tubular epithelium and all the way to the distal nephron by paracrine mediators, such as TNFα [117]. Gomez and colleagues hypothesize that this inflammatory alarm cascade triggers tubular cells to suppress energy turnover and energy-consuming activities and to prioritize processes necessary for cell survival [16]. This is achieved by down-regulating mitochondrial metabolism, redirecting energy consumption, recycling cellular organs, and inhibiting cell division; in other words, the aim is to prevent energy imbalance and DNA damage potentially activating apoptotic and necrotic signaling pathways. The mitochondria orchestrate the switch to "hibernation": their diminished adenosine triphosphate (ATP) production triggers pathways that lead to cell division inhibition, namely cell-cycle arrest [118,119]. Furthermore, mitophagy activates as early as 3 h after cecal ligation and puncture in an experimental sepsis model [120], reducing energy consumption and enabling protein and lipid recycling to produce energy.

It is largely unclear how the tubular cell energy-saving mode links with markedly decreased GFR. One suggested mediating mechanism is the tubuloglomerular feedback system [16]. As a result of cellular energy conservation, energy-consuming ion transporters are inhibited. For instance, tubular sodium and chloride reabsorption decreases [120]. Consequently, NaCl increases at the *macula densa*, and the tubuloglomerular feedback system mediates the afferent arteriole vasoconstriction, resulting in a GFR decrease [16]. Evidently, lower GFR further saves energy by reducing the electrolyte reabsorption workload in tubular cells.

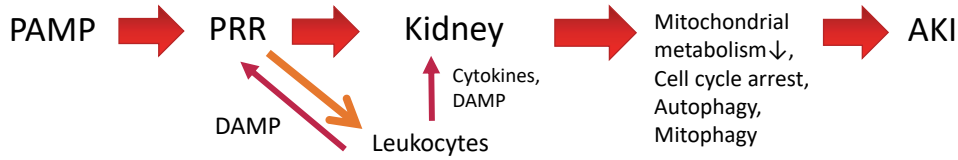


Figure 2 A modified schematic illustration of development of septic AKI suggested by Gomez et al. Shock 41(1):3-11 (2014).

AKI, acute kidney injury; DAMP, damage-associated molecular pattern; PAMP, pathogen-associated molecular pattern; PRR, pattern recognition receptor
Figure by the author.

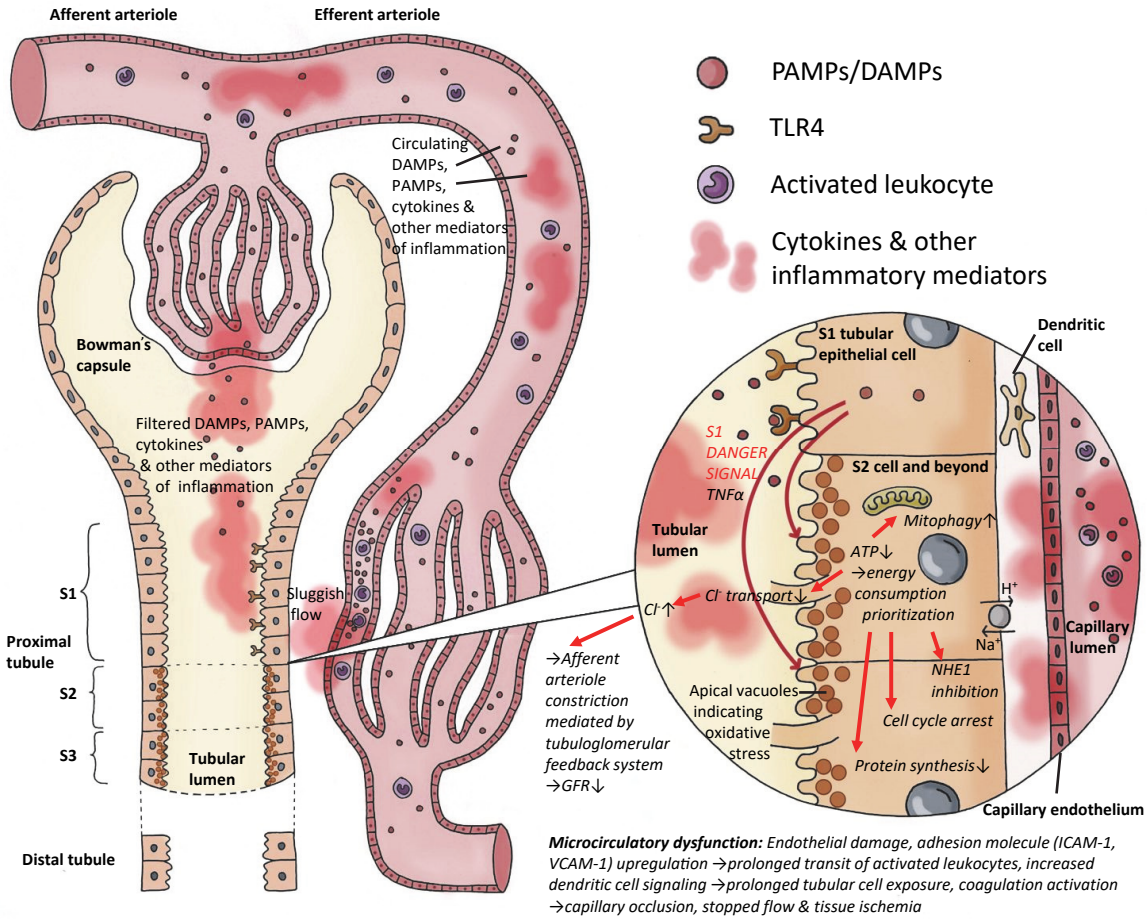


Figure 3 An illustration of renal tubular mechanisms contributing to septic AKI.

ATP, adenosine triphosphate; DAMP, damage-associated molecular pattern; GFR, glomerular filtration rate; ICAM-1, intercellular adhesion molecule 1; NHE1, sodium/hydrogen exchanger 1; PAMP, pathogen-associated molecular pattern; TLR4, toll-like receptor 4; VCAM-1, vascular cell adhesion molecule 1; TNFα, tumor necrosis factor alpha

Figure by Pauliina Pitkänen, based on Gomez et al. Shock 41(1):3-11 (2014).

Unfortunately, little is known of the mechanisms causing the diverse metabolic derangements observed in septic AKI. In sheep with experimental septic shock and severe AKI, crude renal function markers such as RBF, oxygen consumption, and renal lactate flux remained unchanged [84]. It has been shown, though, that inflammatory mediators downregulate the expression and inhibit the activity of ion channels and pumps involved in renal solute reabsorption [121,122]. The consequences are diverse, and the net effect varies, but, for instance, chloride excretion impairment may manifest clinically as hyperchloremic acidosis. Another interfering mechanism, found in rodents with LPS-induced sepsis, is that LPS inhibits the Na^+/H^+ exchanger 1. As a result, bicarbonate re-absorption is blocked in the loop of Henle, which may cause acid–base balance disturbances [123]. However, interpretation of acid–base balance derangements accompanying critical illness is complex because acidifying and alkalizing factors may coexist and influence an AKI patient's blood gas analysis results. The actual cellular mechanisms that lead to vast UO and CrCl changes in human septic AKI are probably numerous, but still poorly known.

To summarize the current understanding of septic AKI, the tubular cell apparently adjusts its metabolism, adapting to the hostile inflammatory environment in order to survive until the conditions improve. This happens at the expense of tubular function that is largely sacrificed—at least transiently. The suggested mechanisms of this emerging theory are illustrated in Figures 2 and 3.

2.4.6 Organ cross-talk

AKI is a syndrome probably affecting most, if not all, organs; interactions with at least the heart, lung, brain, liver, gut, and spleen have been reported to date [124]. According to the literature on acute illness, the kidney and the lung are strongly inter-related. Although mere oliguria in AKI could cause pulmonary fluid accumulation and consequent lung injury, experimental and clinical data suggest that the interaction between the kidney and the lung is mediated by the circulating proinflammatory cytokine IL-6 [125-128]. As an example, a bilateral nephrectomy mouse model demonstrated increased IL-6 levels and associated pulmonary dysfunction following acute loss of kidney function [125]. Another murine AKI study found that, after bilateral nephrectomy or ischemic insult, circulating IL-6 upregulates endothelial chemokine expression leading to neutrophil infiltration into lung tissue [126]. Likewise, Andres-Hernando and colleagues showed that murine ischemic kidney injury causes lung inflammation [127]. Seven days after the experimental insult, the researchers detected elevated circulating IL-6 and pulmonary neutrophil accumulation

with increased MPO activity—even among study participants whose sCr was normal [127]. To conclude, inflammation and neutrophil accumulation appear to be the common denominators in kidney and lung injuries.

The interaction between the kidney and the lung is presumably bidirectional. Analogically to well-known cardiorenal and hepatorenal syndromes, patients with pneumonia or acute respiratory distress syndrome commonly develop AKI [13,129], and mechanical ventilation is a known risk factor for AKI [130].

2.5 Biomarkers of septic AKI

AKI is currently defined using two functional biomarkers, sCr and UO [45]. Besides age, gender, muscle mass, and diet, changes in the volume of distribution could influence Cr values used to detect and stage AKI [47]. Since changes in the classical markers Cr and UO are delayed and their sensitivity and specificity for true tissue-level injury are low, an intense search for earlier and more sensitive markers, kidney stress or injury biomarkers, has been ongoing for a couple of decades. Ideally, a biomarker for AKI should be measurable by a rapid, cost-effective, highly sensitive and specific assay utilizing easily available samples (blood or urine). The ideal biomarker would have dynamically changing levels that correlate with disease progression/improvement and prognosis. Because only supportive care exists for AKI, and treating symptoms with earlier RRT has not brought survival benefit [131], biomarkers are directed predominantly for risk prediction after insult, AKI prevention or early diagnosis, and possibly for evaluation of AKI progression and recovery [64].

However, the clinical performance of suggested AKI biomarkers has been far from perfect, and their value beyond conventional clinical evaluation and routine laboratory data has been questioned [132]. Many candidate markers have succeeded well among children, who have less comorbidities than adults. Some have predicted AKI satisfactorily in the perioperative period when patients have a known time-point of kidney insult and sampling can be timed accordingly. Sepsis patients, however, are admitted to the hospital at variable disease phases and the exact time of the kidney insult is often unclear—as is the optimal time for drawing a biomarker sample [133]. Moreover, the flourishing systemic inflammation and neutrophil activation complicates AKI biomarker interpretation. Suggested biomarkers for septic AKI include NGAL, kidney injury molecule 1 (KIM-1), sTREM-1, and cell cycle arrest proteins TIMP-2 and IGFBP-7 [134,135]. Table 5 compares these biomarkers.

Table 5 Characteristics of some of the most studied AKI biomarkers suggested for diagnosing septic AKI. Sensitivity, specificity and AUROC data for NGAL, KIM-1 and TIMP-2 x IGFBP-7 from meta-analyses by Albert et al. [136], Geng et al. [137], and Zhang et al. [138].

AKI biomarker	**NGAL**	**KIM-1**	**sTREM-1[a]**	**TIMP-2 x IGFBP-7**
Source	3 molecular forms, of which 2 produced by damaged renal tubular cells and all 3 by activated neutrophils	released into urine from damaged proximal tubular cells	soluble form of a surface receptor of neutrophils, monocytes, and macrophages	kidney stress markers of cell cycle arrest
Sample	plasma or urine	urine	plasma or urine	urine
Marker of stress(S)/ damage(D)	D	D	?	S
Suggested clinical use in AKI				
Prediction	-	+	+	+
Diagnosis	+	+	+	+
Severity	+	+	-	+
Clinical performance in diagnosing AKI				
Sensitivity (%)	66 (plasma) * 56 (urine) *	74*	94**	89*** 45****
Specificity (%)	73 (plasma) * 71 (urine) *	84*	76**	48*** 93****
AUROC	0.77 (plasma)* 0.74 (urine) *	0.62*	0.75 to 0.79 (plasma) 0.71 to 0.92** (urine)	0.75*** 0.84****
Cost effectiveness data available	-	-	-	+/- (limited data in hospital population)

[a] no meta-analyses of AKI prediction or diagnosis available, data from two small sepsis studies[34,139]
* cutoff by Youden index, ** cutoff 69.04 pg/ml, *** cutoff 0.3 $(ng/ml)^2/1000$, **** cutoff 2.0 $(ng/ml)^2/1000$

AUROC, area under the receiver operating characteristic curve; IGFBP-7 insulin-like growth factor-binding protein 7; KIM-1, kidney injury molecule 1; NGAL, neutrophil gelatinase-associated lipocalin; sTREM-1, soluble form of the triggering receptor expressed on myeloid cells 1; TIMP-2, tissue inhibitor of metalloproteinases 2

2.5.1 Proinflammatory mediators IL-6 and IL-8

As pointed out above, the current view is that septic AKI develops as a result of dysregulated inflammation, manifesting with a "cytokine storm" [16]. One of the proinflammatory cytokines, IL-6, is secreted by macrophages as a response to PAMPs bound to pattern recognition receptors on their cell surface. IL-6 mediates fever and acute phase reaction and stimulates neutrophil production in the bone marrow, among other things. High IL-6 plasma concentration is strongly associated with septic AKI and elevates as AKI stage grows, along with mortality [18,19]. *In vitro* studies have shown that circulating IL-6, after binding with the soluble IL-6 receptor, induces IL-8 production by endothelial cells [126]. IL-8 is a powerful neutrophil chemoattractant, involved in neutrophil activation, infiltration into tissues, and phagocytosis, and has been reported to be associated with slower kidney recovery and higher mortality among RRT-treated critically ill patients [140]. Some studies have shown markedly high IL-6 and IL-8 levels even among AKI patients with less severe infection and no sepsis at all, indicating that AKI is a stronger determinant than sepsis when it comes to IL-6 and IL-8 elevation [13,141].

2.5.2 Activin A

Activin A is a member of the transforming growth factor-β superfamily. Besides functions involving cell proliferation, differentiation, and apoptosis, activin A is a multifunctional cytokine involved in many inflammatory diseases. LPS challenge sepsis experiments show that activin A rises in the serum even earlier than IL-6 [23,24,142]. Neutrophil cytosol is the main source of rapidly released activin A [20-22,143], which probably explains its early intravascular peaking. Neutrophils are not only the origin of activin A but also the targets of it as activin A has been shown to increase neutrophil phagocytic function and oxidative burst in mice [108]. Correlation with human sepsis severity and mortality has been reported [144], but, to our knowledge, activin A involvement in septic AKI has not been previously investigated.

2.5.3 Neutrophil granule markers HBP, MPO, and NGAL

HBP, a member of the serine protease family, is a neutrophil granule protein. HBP is released into circulation from secretory vesicles when polymorphonuclear leukocytes are stimulated by certain bacterial structures, and is markedly elevated in septic AKI [27]. It mediates endothelial hyperpermeability [27,145,146]. The HBP rise takes place at least 12 h before hypotension onset and up to 24 h before OD in sepsis patients [29,147]. MPO, a traditional neutrophil activation marker, is, unlike HBP, predominantly released from neutrophil azurophilic

granules after neutrophil infiltration into tissues [26]. Therefore, MPO tissue concentration reflects the magnitude of neutrophil infiltration in that tissue. Renal MPO levels cannot be directly measured, but urine samples are easily obtainable.

NGAL is a widely studied biomarker for renal tubular injury, used to detect AKI, and to monitor AKI evolution and outcome [148,149] (Table 5). It is released from specific granules of activated neutrophils during bacterial infection and systemic inflammation, but has numerous other functions and sources, including kidney, respiratory, and gastrointestinal tract epithelia. Sepsis is known to raise NGAL levels, which may confound measurements for septic AKI [150,151]. Another problem is that NGAL is elevated in CKD [152]. Despite these pitfalls, the assay is already clinically used in several countries. Either urine or plasma samples can be analyzed, but uNGAL may have higher specificity for AKI. However, the optimal cutoff values are unknown and NGAL performance in septic AKI is unclear.

2.5.4 KIM-1

KIM-1 is a type 1 transmembrane glycoprotein of the proximal tubular cells and a receptor for phosphatidylserine, a signaling agent that identifies apoptotic cells to be phagocytosed. Elevated KIM-1 levels are detected in toxic and ischemic kidney injury [153,154]. Performance figures of KIM-1 are listed in Table 5. A study of 150 sepsis patients reported an area under the receiver operating characteristic curve (AUROC) of 0.864 for urine KIM-1 to diagnose AKI 6 h after ICU admission, but with earlier samples, AUROCs were under 0.500 [155].

2.5.5 sTREM-1

Triggering receptor expressed on myeloid cells 1 is a member of the immunoglobulin superfamily, found on neutrophil and monocyte surfaces, and involved in the inflammatory reaction. The soluble form of this transmembrane receptor, sTREM-1, is a suggested biomarker of disease severity in sepsis [139]. A few small sepsis studies have reported that serum and urine sTREM-1 is associated with AKI [34,139] , AUROCs presented in Table 5.

2.5.6 Cell cycle arrest markers: TIMP-2 and IGFBP-7

Cell cycle arrest markers have been under investigation because they might offer an early warning of evolving AKI. Table 5 shows the characteristics of TIMP-2 and IGFBP-7 combined. These two have also been promising to predict septic AKI [35]. In a study analyzing AUROCs and risk prediction improvement

compared with clinical variables, a combination of TIMP-2 and IGFBP-7 was superior to any previously suggested biomarker in critically ill patients, including those with sepsis [156]. This test is currently commercially available for evaluating patient's risk for impending AKI. TIMP-2 and IGFBP-7 are probably useful as "kidney stress markers" but not as actual injury markers, and the response to a furosemide stress test may be better than cell cycle arrest markers, or any laboratory marker, to predict AKI evolution [157].

2.5.7 Predicting AKI progression: furosemide stress test

The furosemide stress test is a clinical tool suggested for AKI progression evaluation. Intravenous furosemide (1.5 mg/kg for those previously on furosemide medication and 1.0 mg/kg for those without) is given to a patient of interest. Urine output >200 ml over 2 hours after furosemide administration is considered responsiveness [158]. According to observational studies, unresponsive patients have an increased risk for progression to stage 3 AKI, an increased need for RRT, and increased mortality [157-160].

2.5.8 Statistical evaluation of biomarkers for clinical use

A biomarker measurement is justified only if it improves clinical judgement and outperforms traditional markers, such as Cr and UO. To assess biomarker performance before its clinical implementation, the first step is to define the population of interest. Second, one must set a clinically meaningful goal for the biomarker: Is it early diagnosis, prediction of a clinically relevant outcome, kidney function monitoring, or something else? Aiming at early AKI detection probably emphasizes test sensitivity over specificity, whereas AKI monitoring (for instance, response to therapy) would require a high specificity. Third, pre-existing, competing risk models must be taken into consideration. Fourth, appropriate statistical tools and model evaluation tests must be chosen. For instance, D'Agostino has described a step-by-step formula to follow [161]. To start, the statistical significance and the relative risk or hazard ratio of the new test must be assessed in a multivariate model which adjusts for the known risk factors and other relevant variables. Provided that the new test is statistically significant and has a clinically meaningful relative risk/hazard ratio, the test's discriminative ability—that is, its ability to rank individuals correctly in order of their risk and to separate those developing the outcome event from those who will not—should be tested. An established means to do this involves calculating the AUROC. Next, test calibration, completed using the chi-squared test, describes how the predictions of a model match the actual observed rates.

In the literature, novel AKI biomarkers are often analyzed using AUROC analyses alone. Unfortunately, calculating the AUROC does not address a biomarker's incremental value compared with conventional clinical examination and laboratory testing. Thus, reclassification through the prediction model that includes the new biomarker can be evaluated using recently introduced category-free net reclassification improvement (cfNRI) and integrated discrimination improvement (IDI) [162]. However, these metrics have been criticized for being too cryptic to interpret and even misleading [163,164]. With risk assessment plots (RAPs), cfNRI and IDI values are visualized, and model improvement or non-improvement in patients with and without the outcome event become easily observable across varying risks of the event [165]. When evaluating a proposed biomarker, the objective is to assess the clinical usefulness of the model including the new marker—that is, to compare the old and the new prediction model to find out if a net benefit exists when the new biomarker is added. A novel method for this comparison is the decision curve analysis (DCA) [166,167], described in detail in the methods section.

While it is evident that AUROCs alone are insufficient to evaluate biomarkers for clinical use, the novel statistic tools discussed above have their shortcomings as well and cannot replace sensitivity and specificity measures or c-statistics. If a proposed biomarker does not have a clinically meaningful hazard ratio or good enough discrimination and calibration in the population in question, further clinical usefulness evaluation with advanced statistical tools is obviously not indicated. In conclusion, to scrutinize a novel biomarker, a stepwise and thorough approach involving several statistical tiers is mandatory [168,169].

2.6 Outcome of septic AKI

A global multicenter study reported hospital mortality from sepsis to be as high as 35%, although variations between countries and even between hospitals were significant [36]. Despite recent survival improvements [170,171], absolute mortality is growing worldwide as sepsis incidence is rising [69,172]. Sepsis and AKI are a particularly deadly combination: concomitant AKI worsens sepsis prognosis significantly [173] and seems to be an independent predictor of death [19,174,175]. Studies have shown that an ICU sepsis patient with AKI has up to 6–8 times higher risk for in-hospital death than those without AKI [176,177]. A Finnish national multicenter study found that 90-day mortality was 24.7% in severe sepsis patients without AKI and 38.1% in those with AKI [69]. Moreover, the mortality increased along with septic AKI severity: the 90-day mortality was 31.4% in sepsis patients with KDIGO stage 1 AKI, 46.2% in patients with KDIGO stage 3 AKI and 49.3% in those treated with RRT for septic AKI. The corresponding

hospital mortality figures were 19.1% (KDIGO1) to 42.8% (RRT) [69]. Importantly, overall hospital mortality is higher in septic AKI compared with AKI of another etiology, possibly due to inflammation-induced extrarenal OD that worsens the prognosis [178,179].

In countries with adequate critical care and other health-care facilities, an increasing number of septic AKI patients will survive. Septic AKI is a syndrome of acute kidney function impairment and organ damage not only causing acute electrolyte and acid–base disturbances, deranged fluid balance regulation, and hindered excretion of waste products, but also long-term adverse outcomes and comorbidity. Although many consequences of AKI can be controlled and treated with RRT, earlier RRT initiation has not improved survival among ICU AKI patients [131,180,181]. AKI severity depends on the extent of acute tissue injury superimposed on underlying organ reserve. Septic AKI is often reversible, but long insult duration and poor renal reserve may limit renal function restoration [182,183]. When AKI of at least stage 1 persists after 7 days from the initiating event, the criteria for acute kidney disease are met. In case of non-recovery at three months from the initiating event, CKD diagnosis can be made. A fact to acknowledge is that sCr may not be the ideal AKI recovery marker for ICU patients who often suffer from muscle wasting that lowers creatinine values. Even in the fortunate case of complete functional recovery, characterized by sCr normalization, a history of AKI still strongly predisposes the individual to future AKI relapses [184,185], CKD, end-stage renal disease, and non-renal adverse outcomes such as cardiovascular disease [7,173,185-188].

2.7 Prevention and future treatment of septic AKI

According to a meta-analysis comprising 154 studies and over 3 million participants, AKI complicates the hospital stay of 1 in 5 adults [189]. Although no specific therapies for established AKI exist, it has been claimed that as many as 20–30% of the cases might be preventable if increased risk was recognized early enough [190]. Machine learning risk assessment tools may help in finding vulnerable patients [191], and nephrology rapid response teams, triggered by AKI risk assessment and TIMP-2 and IGFBP-7 monitoring, have been suggested [192]. To prevent septic AKI specifically, the first step is to recognize sepsis earlier. Accordingly, campaigns aim at raising concern among professionals as well as laymen. Inside hospitals, developing medical emergency teams and associated criteria for escalating care enables prompt recognition and timely reaction to emerging sepsis. To date, septic AKI prevention has leaned on the early initiation of appropriate antibiotic therapy, source control and hemodynamic stabilization. Few clinical studies have tested pharmacologic therapies aiming

specifically to prevent or mitigate septic AKI, but human recombinant alkaline phosphatase and angiotensin II are two potentially beneficial agents [193].

Since 2019, immunomodulation has returned to the limelight as the pandemic caused by severe acute respiratory syndrome coronavirus-2 [194] has evoked enormous research resource mobilization across the scientific community. In this novel coronavirus disease 2019 (COVID-19), severe hypoxic respiratory dysfunction is the clinical hallmark of those who end up in intensive care—immune response dysregulation being the most important disease severity denominator. This observation has directed treatment attempts toward immunomodulatory drugs [195]. Although COVID-19 pathophysiology is somewhat different from bacterial sepsis, there are noticeable similarities: hyperinflammation seems to be associated with severe disease and multi-organ failure in COVID-19 as well [196]. While early effective antibiotic therapy and source control remain essential to improve sepsis prognosis and reduce AKI [197], the evidence gained from immunomodulatory COVID-19 treatment may advance future treatment of sepsis and associated OD, including AKI.

2.7.1 Alkaline phosphatase

Human recombinant alkaline phosphatase, an endogenous dephosphorylating enzyme, has shown promise in treating inflammation in septic AKI [198]. Alkaline phosphatase was recently shown to improve endogenous CrCl as well as septic AKI 28-day and 90-day survival [199]. Alkaline phosphatase cleaves phosphate groups from LPS [200] and other PAMPs and DAMPs. Dephosphorylated endotoxin still binds to TLR4 but fails to activate the signaling cascade, thus acting as an antagonist to TLR4-mediated signaling. Alkaline phosphatase also inhibits inducible nitric oxide synthase and thus presumably restrains nitric oxide mediated caspase-3 activity preserving peritubular microcirculation [201]. As evidence of the suggested mechanisms, animal studies have demonstrated inflammatory response attenuation (for instance, lower IL-6 and nitric oxide levels) following alkaline phosphatase treatment [202,203]. Another target of alkaline phosphatase is extracellular ATP, which is released by stressed renal cells and acts as a DAMP. Alkaline phosphatase dephosphorylates ATP yielding adenosine, which is a mediator in *macula densa* control of GFR via the tubuloglomerular feedback system [204].

2.7.2 Angiotensin II

Angiotensin II is part of the renin-angiotensin-aldosterone system that activates in hypotensive states. Angiotensin II binds to a G-protein receptor in blood vessels, causing vasoconstriction. It also increases tubular NaCl

reabsorption and water retention, aldosterone and vasopressin secretion, and sympathetic activity. A *post hoc* analysis of the Angiotensin II for the Treatment of Vasodilatory Shock 3 (ATHOS-3) trial reported improved 28-day survival and less RRT -dependency at day 7 among RRT-treated patients who received angiotensin II for refractory vasodilatory shock [205]. However, this observation has not been replicated, and the ATHOS-3 trial limitations regarding internal and external validity warrant further studies before implementing this treatment.

2.7.3 Corticosteroids

Corticosteroids act as non-specific inflammation attenuators and are utilized in various states of acute and chronic inflammation. The well-known corticosteroid side -effects include hyperglycemia, hypernatremia, increased risk for ICU-acquired weakness and susceptibility to superinfections. Unlike in COVID-19 [206,207], the benefit versus harm of corticosteroids is continuously debated in sepsis. A series of contradicting trials and alternating meta-analyses have been published over the years. Yet, the most recent pooled data on corticosteroids in septic shock indicate a 28-day survival benefit, shorter time to shock resolution, and a lower SOFA score on day 7 [208]. The latest guidelines by the Surviving Sepsis Campaign suggest hydrocortisone in septic shock with ongoing vasopressor therapy [209].

2.7.4 Blocking the "cytokine storm"

Tocilizumab, a humanized monoclonal antibody that inhibits IL-6 by blocking the IL-6 receptor, is an immunosuppressive drug for rheumatoid arthritis, juvenile idiopathic polyarthritis, giant cell arteritis, and severe cytokine release syndrome related to chimeric antigen receptor T-cell therapies. High IL-6 levels in broncho-alveolar lavage fluid and sputum are associated with severe COVID-19 [210]. Following results indicating reduced mortality and reduced need for invasive ventilation from two large COVID-19 trials [211,212], the World Health Organization now strongly recommends systemic IL-6 inhibitors, added to corticosteroid treatment, for life-threatening COVID-19 [213]. Apparently, certain gene variants of the IL-6 inflammatory pathway are associated with the most severe COVID-19 forms [214], supporting the treatment strategy of preventing IL-6 action. A similar approach has not improved survival among non-selected sepsis patients thus far, but phenotyping patients can help in identifying hyperinflammatory individuals [81] who might benefit from therapies suppressing the IL-6 pathway [215]. Concurrently, the potential harms of immunomodulatory

therapies, particularly the increased risk of secondary infections, must be considered carefully [216].

Another drug for rheumatic and autoimmune diseases, a recombinant receptor antagonist of IL-1α and IL-1β, anakinra, is currently studied as a potential COVID-19 medication. Like IL-6, IL-1 takes part in the inflammatory response to lung injury [217] and "cytokine storms". Anakinra is used, off label, to treat hyperinflammatory conditions [218]. A recent meta-analysis of non-randomized cohort studies indicated significant reduction in COVID-19 mortality and in need for mechanical ventilation [219]. Although randomized controlled trials studying anakinra in sepsis have failed to demonstrate reduced mortality [220,221], a subgroup analysis indicated improved survival among sepsis patients with hyperinflammation [222].

To conclude, immunomodulatory agents dampening the "cytokine storm" seem to perform better in severe COVID-19 than in sepsis. There may be several explanations for this. Obviously, COVID-19 is caused by one virus whereas sepsis by various bacteria or fungi. Perhaps more importantly, the study population of a trial investigating a single viral disease is inevitably more selected and homogenous than the corresponding sepsis trial population. Another factor explaining response to immunomodulation may be that COVID-19 patients expressing the most severe respiratory dysfunction and hypoxia also have the highest cytokine levels [196]. Furthermore, the timing of hospital admission and, thus, initiation of medication, is highly constant with COVID-19: dyspnea requiring hospitalization usually develops at approximately one week from the first symptoms [223]. This time point is considered safe to introduce immunomodulatory drugs that would potentially weaken the initial response to the microbial invader. Choosing the right patient phenotypes and the optimal timing for immunomodulation is probably more laborious in the more diverse sepsis syndrome but might bring significant progress.

3 AIMS OF THE STUDY

The primary aim of this study was to explore suggested inflammatory mechanisms of septic AKI and to evaluate some present and emerging septic AKI biomarkers in a clinical intensive care setting.

The specific targets were:

1. To evaluate the usefulness of adjusting plasma Cr for fluid balance: To discover if it influences the ICU AKI incidence and if 90-day mortality differs between patients who changed category from non-AKI to AKI after adjustment and those who did not (I).

2. To study the association of proinflammatory cytokines and neutrophil activation markers with septic AKI in critically ill sepsis patients (II).

3. To study the temporal changes in proinflammatory cytokine levels and intravascular neutrophil activation in relation to OD onset in critically ill patients with early sepsis (III).

4. To evaluate, with novel statistical instruments, whether uNGAL adds value to risk models based on routine clinical and laboratory evaluation in predicting AKI, RRT or 90-day mortality in critically ill sepsis patients (IV).

4 PATIENTS AND METHODS

4.1 Patients

All patients in Studies I–IV were participants in the FINNAKI study [4], which was a prospective observational national study conducted in 17 Finnish ICUs. Between 1 September 2011 and 1 February 2012, all emergency admissions and elective admissions with an expected ICU stay >24 h were included. The exclusion criteria were as follows: 1) Age under 18, 2) Readmitted patient who had received RRT during the previous admission, 3) Elective patient treated for <24 h if discharged alive, 4) Patient on chronic dialysis, 5) Organ donor, 6) No permanent residency in Finland or insufficient language skills, 7) Transfer from another ICU if patient had already participated in the study for 5 days, 8) Patient in intermediate care.

Patients of **Study I** originated from the FINNAKI study. We excluded patients having stayed in ICU for <24 h, those transferred to another ICU during the first 5 days, those with missing fluid input/output or weight data, and those treated with RRT prior to ICU admission.

Study II included 182 FINNAKI study patients who fulfilled the ACCP/SCCM criteria [37] for severe sepsis at any time between 24 h preceding admission and the second calendar day in the ICU. To achieve this number, 237 consecutive patients were screened from the end of the FINNAKI study period backwards. Patients not having both admission and 24 h plasma samples available were excluded.

In **Study III**, we included all Study II patients who had a recorded time label for the first emerging sepsis-associated OD.

In **Study IV**, we investigated a sepsis patient subpopulation from the FINNAKI-NGAL -study [32], which included a random patient sample from the first half of the original FINNAKI study. We included only patients who fulfilled the most recent Sepsis-3 criteria [41] during the first three ICU days. Flow charts of Studies I–IV are shown in Figure 4.

Studies I–IV were approved by the local Ethics Committee of the Helsinki University Hospital. A deferred consent was granted to include patients immediately upon ICU admission and to obtain samples promptly. This was considered appropriate as the studies were observational and included no interventions other than collecting a small amount of blood and urine. When deferred consent was applied, a written informed consent was obtained from the patient or next of kin as soon as possible.

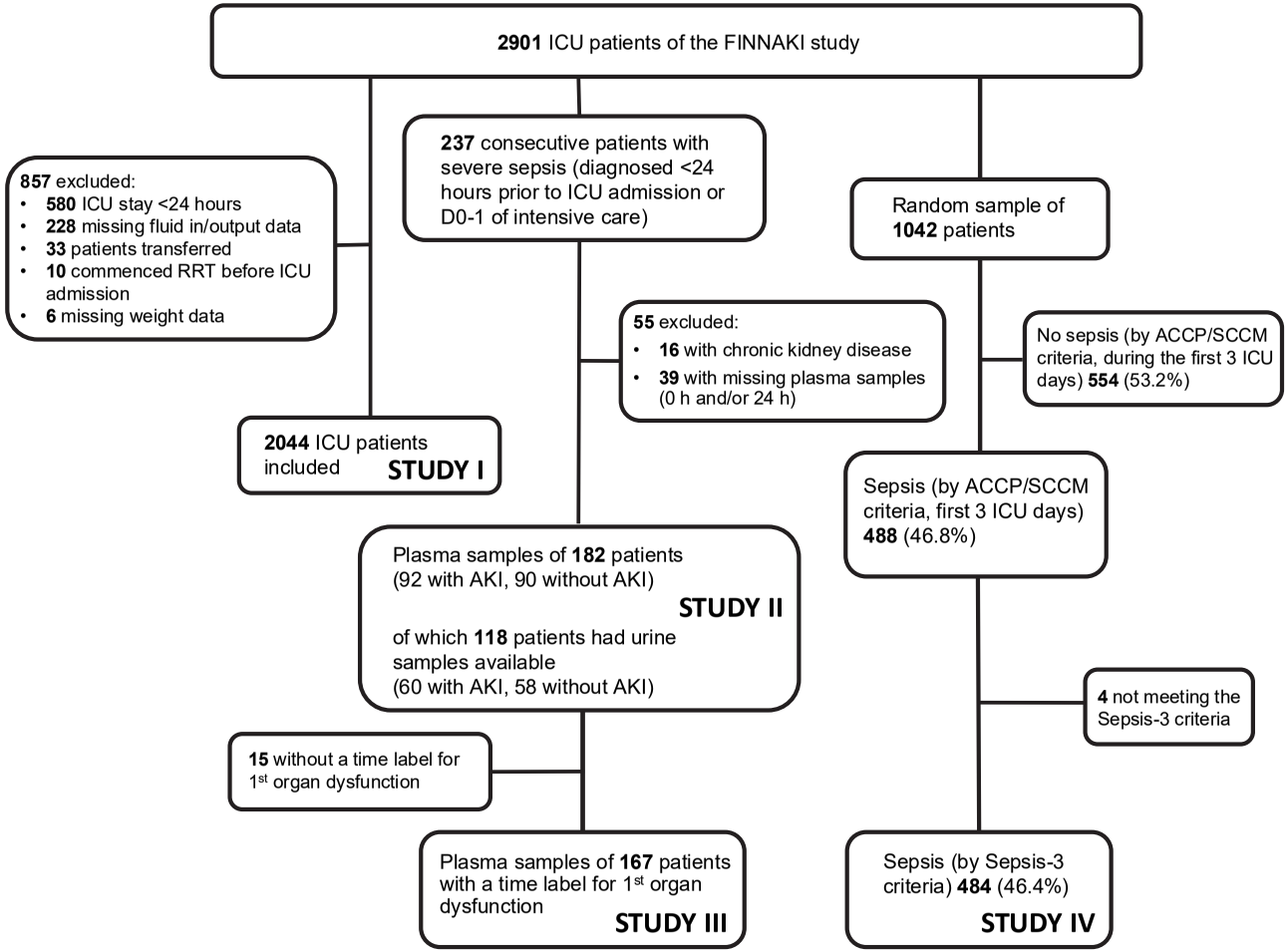


Figure 4 Flow chart of Studies I–IV.

ACCP/SCCM, American College of Chest Physicians/Society of Critical Care Medicine; FINNAKI study, FINNish Acute Kidney Injury study; ICU, intensive care unit; D, day; RRT, renal replacement therapy. Figure by the author.

4.2 Data collection

Studies I–IV utilized prospectively collected data from the FINNAKI study [4]. Clinicians filled in a standardized case report form on patients' admission to ICU and daily thereafter until discharge or day 5. Comorbidities, present health status, certain pre-determined medications and laboratory test results, possible risk factors for AKI, information on operative treatment, daily fluid input and output, AKI, and RRT were recorded in the case report form (CRF).

In Study I, we calculated fluid balance by subtracting daily total fluid output (urine output, ultrafiltration, losses to drains and gastrointestinal tract, and a surrogate for evaporation) from daily total input (intravenous crystalloids, colloids, blood products, drug infusions, nutrition, and oral intake). To adjust Cr measurements for the fluid balance, we used the following previously introduced formula [58,224]:

adjusted Cr = Cr x [(0.6 x patient weight) + cumulative balance in liters] / (0.6 x patient weight).

The fluid balance used for Cr adjustment was calculated as the cumulative fluid balance from ICU admission until each Cr measurement. For the analyses, we chose the highest unadjusted Cr and the highest adjusted Cr of the first 5 ICU days.

Study III utilized the CRF originally created for the FINNAKI study, with screening for severe sepsis and OD 24 h preceding admission, on admission, and each day until ICU day 5. Data collection has been described previously [69]. The onset of septic shock and every new OD (hh:mm) was recorded by the attending clinician according to the following pre-defined criteria:

1. Hypotension: a) systolic arterial pressure ≤90 mmHg, or reduction of over 40 mmHg from baseline for over 1 h, or b) hypotension not responding to intravenous fluids after 1 h infusion and requiring inotrope/vasopressor treatment.

2. Signs of hypoperfusion: lactate above the local laboratory reference value, or oliguria, or acutely altered mental status.

3. Acute respiratory dysfunction: ratio of the partial pressure of oxygen in arterial blood to the inspired oxygen fraction <200 mmHg, if respiratory dysfunction only, and <250 mmHg, if other ODs present.

4. Acute kidney dysfunction: UO ≤0.5 ml/kg for 1 h despite adequate fluid resuscitation.

5. Acute cardiovascular dysfunction: systolic arterial pressure ≤90 mmHg or mean arterial pressure (MAP) ≤70 mmHg for at least 1 h.

6. Acute hematological dysfunction: thrombocytes ≤80 E9/l or ≥50% decrease in 3 days.

7. Unexplained metabolic acidosis: pH ≤7.30, or base excess ≤-5 and lactate >1.5 times normal likely to be caused by infection.

Admission diagnoses, physiologic data including hourly UO, and prognostic (acute physiology and chronic health evaluation II, APACHE II; simplified acute physiology score II, SAPS II) as well as disease severity scores (SOFA) were collected from the Finnish Intensive Care Consortium database. This database, maintained by Tieto Ltd, Helsinki, Finland, and described in the original FINNAKI study report [4], consists of routinely collected benchmarking data from Finnish ICUs. The data are mostly automatically transferred from electronic medical records, laboratory systems, patient monitors, and ventilators, but validated by automated filters and by trained personnel.

4.2.1 Sepsis definition (II–IV)

Sepsis definition has evolved in recent years. At the time of the FINNAKI study enrollment, the ACCP/SCCM criteria [37] were used to screen sepsis. To have severe sepsis, which was the inclusion criteria in Studies II–III, a patient had to fulfill all three of the following criteria:

1. Verified or strongly suspected infection

2. SIRS, that is, at least two of the following:
   a. core temperature ≥38°C or ≤36°C
   b. heart rate ≥90/min
   c. respiratory rate ≥20/min or partial pressure of carbon dioxide ≤4.3 kPa in arterial blood, when breathing spontaneously, or ventilator support
   d. white blood cell count ≥12.0 E9/l or ≤4.0 E9/l or >10% immature neutrophils

3. At least one OD likely to be caused by infection:
   a. hypotension not responding to fluid therapy
   b. signs of hypoperfusion (lactatemia, oliguria, acute change in level of consciousness)
   c. acute respiratory dysfunction
   d. AKI
   e. acute liver failure
   f. metabolic acidosis
   g. disseminated intravascular coagulation defined by International Society on Thrombosis and Haemostasis criteria [225]

To detect OD, we used the SOFA score [38]. In Studies II–III, the attending clinician was responsible for deciding whether or not a study patient had infection-related OD. In Study IV, sepsis was defined *post hoc* according to the latest Sepsis-3 definition [41] as a life-threatening OD caused by a dysregulated host response to infection. The criteria for sepsis included suspected or documented infection and an acute increase of ≥2 SOFA points, reflecting OD [38].

4.2.2 AKI definition

We defined and staged AKI according to KDIGO criteria [45], including plasma Cr, UO, and RRT criteria. In addition, we used fluid balance-adjusted Cr for AKI diagnosis and staging in Study I. The last available plasma creatinine value from the preceding year up to one week before ICU admission was used as the baseline value. If not available, we estimated the baseline creatinine value using the MDRD equation assuming a GFR of 75 ml/min/1.73 m^2 [52]. The highest KDIGO stage of the observation period was chosen for each patient's final KDIGO stage. In Study IV, we defined KDIGO stage 2–3 AKI as "severe" AKI.

4.2.3 Outcomes

Throughout the observation period, given RRT was recorded daily in the CRF. ICU length of stay data were collected from the Finnish Intensive Care Consortium database. 90-day all-cause mortality data for Studies I, II, and IV were obtained from the Finnish Population Register Center.

4.3 Blood and urine samples

Plasma Cr samples were obtained daily as part of ICU routine laboratory testing. Blood samples for Studies II–III were drawn from the peripheral arterial cannula into ethylenediaminetetraacetic acid tubes upon ICU admission and 24 h later. The admission samples were centrifuged and aliquoted immediately after ICU admission or by 2 hours at the latest. Storage was at -80°C until assayed.

We collected urine samples at the time of ICU admission in Study II. For Study IV, urine samples were obtained on admission, and at 12 h and 24 h from admission. All urine samples were centrifuged and aliquoted as soon as possible and stored at -80°C until assayed.

4.4 Laboratory assays

We used commercially available kits and conducted all laboratory assays in duplicate according to the manufacturer's instructions. The individuals who performed the laboratory analyses were blinded to patient records. Daily routine laboratory tests including blood leukocytes, plasma C-reactive protein (CRP), and Cr were analyzed in the local laboratory, HUSLAB.

4.4.1 Activin A (II)

For plasma and urine assays of activin A in Study II, we used a commercial enzyme-linked immunosorbent assay (ELISA) kit (Quantikine®, R&D Systems, Abingdon, UK; detection limit 4 pg/ml).

4.4.2 IL-6 (II–III)

For plasma IL-6 assays in Studies II–III and urine IL-6 assays in Study II, we used a commercial ELISA kit (DiaClone®, Besancon, France; detection limit 2 pg/ml).

4.4.3 IL-8 (II–III)

For plasma IL-8 assays in Studies II–III and urine IL-8 assays in Study II, we used a commercial ELISA kit (Cymax®, Abfrontier, Seoul, South Korea; detection limit 0.3 pg/ml).

4.4.4 MPO (II–III)

For plasma MPO assays in Studies II–III and urine MPO assays in Study II, we used a commercial ELISA kit (BioLegend Inc., San Diego, CA, USA; detection limit 28 pg/ml).

4.4.5 HBP (III)

For plasma HBP assays in Study III, we used a commercial ELISA kit (Axis-Shiled Diagnostics, Dundee, UK; detection limit 5.9 ng/ml).

4.4.6 NGAL (IV)

NGAL ELISA Rapid Kit (BioPorto® Gentofte, Denmark; measurement range 10–1000 ng/ml), with good intra- and inter-assay precision, was used for uNGAL assays in Study IV.

4.5 Statistical analyses

4.5.1 Sample size calculations (II–III)

For power calculation in Study II, previous clinical data only existed for IL-6 concentrations in septic AKI [19]. Additionally, we utilized the results of an unpublished pilot study of 32 sepsis patients, presented later in the results. We aimed at 80% power to detect a 2.5-fold increase in plasma IL-6 concentration between AKI and non-AKI patients. To be on the safe side when previous data for plasma IL-8, Activin A and MPO did not exist, we increased the sample size to 90 per group (AKI and non-AKI). For Study III, we used the IL-6 and IL-8 results of the above-mentioned pilot study and one-way analysis of variance, the parametric equivalent of the Kruskall–Wallis test, to estimate the patient number needed for 80% power. To get the sample size for a non-parametric test, we added 10% to the resulting number of patients, as usual. We could not influence the sample size in Studies I and IV because they were *post hoc* analyses of previously published studies [4,32].

4.5.2 Data presentation and group comparison

We present the data as numbers and percentages, as medians with interquartile range (IQR) or range, or as means with standard deviations, depending on distributions. Point estimates of incidences and AUROCs are presented with 95% confidence intervals (CIs). For study variables with non-normal

distributions, we used non-parametric tests: proportions were compared using Chi square or Fisher's exact tests and bivariate correlations with the Spearman test. For continuous variables, we used the Mann-Whitney U test to compare groups. For comparisons between three or more groups, we chose the Kruskall–Wallis test with pairwise comparison as a *post hoc* test. In Study I, we used Wilson's method for non-paired samples to compare mortality rates between non-paired groups and Student's T-test to compare groups, when appropriate. P-values <0.05 were considered significant except when Bonferroni correction was used, and the p-value was lowered as appropriate.

4.5.3 Logistic regression analysis (II) and multivariable logistic regression analysis (IV)

In Study II, we used logistic regression analysis to test if comorbidities or disease severity contributed to AKI. We entered all covariates at the same time, not stepwise. To construct clinical risk models in Study IV, we conducted multivariable logistic regression analysis entering variables with the strongest associations with outcomes (max 1 per 8 dependent endpoints) simultaneously. We imputed missing values as recommended [226]. Model calibration was tested with Hosmer–Lemeshow goodness of fit.

4.5.4 AUROC (II, IV)

In Studies II and IV, we calculated AUROCs using the method published by DeLong et al. [227]. We presented binomial exact 95% CIs for AUROCs to assess the diagnostic accuracy of the studied biomarkers (II) and the risk models (IV).

4.5.5 Category-free net reclassification improvement (cfNRI), integrated discrimination improvement (IDI), and risk assessment plot (RAP) (IV)

In Study IV, we evaluated the additive predictive power of uNGAL with cfNRI and IDI. In the risk models of Study IV, each patient received a calculated risk probability from 0 (0%) to 1 (100%). We compared the probabilities of the new risk model including uNGAL with the probabilities of the clinical risk model ("old"). Each patient was given a value of either +1 or -1 depending on whether the change in the calculated risk was in the correct direction (higher for those with events and lower for those without) or incorrect direction (lower for those with events and higher for those without), respectively. The $cfNRI_{events}$ is the sum of these values among the patients with the outcome event and $cfNRI_{nonevents}$, correspondingly, the sum of the values among the patients without the outcome

event. The cfNRI is the sum of $cfNRI_{events}$ and $cfNRI_{nonevents}$. We also calculated IDI, which detects both the direction and the quantity of the change between risk models for patients with and without the event, defined as

$$IDI = (IS_{new} - IS_{old}) - (IP_{new} - IP_{old}),$$

where IS is the integral of sensitivity over all possible cut-off values and IP is the integral of "1 minus specificity". We draw RAPs, plotting the portion of patients above a certain calculated risk against the certain risk, to visualize how cfNRI and IDI vary in patients with and without the event, according to the risk of the event.

4.5.6 Decision curve analysis (DCA) (IV)

In Study IV, we conducted DCAs plotting net benefit against threshold probability for the outcomes to illustrate the net benefit of adding uNGAL to the clinical prediction models. Net benefit delineates gained new true-positive results without false-positive results varying according to the chosen threshold probability. This is the probability above which the patient is offered treatment (for instance, ICU admission). A threshold probability is chosen according to the significance of false-negative versus false-positive results. Threshold probability of 0.1 means that we consider the harm of a false-negative result (for instance, denial of a necessary ICU admission) to be 9 times (1 - 0.1/0.1) worse than a false-positive result (unnecessary ICU admission). For more serious outcomes, false-negative results are considered more harmful and the threshold lowered. We calculated test trade-offs to determine the minimum patient number to be tested per one extra true-positive classification.

4.5.7 Software

We used SPSS 22 or 24 software (SPSS Inc., Chicago, IL, USA), MedCalc Statistical Software version 18 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2018), R 3.4.3 and 3.6.1 (R Development Core Team, Vienna, Austria), and Stata 16 (StataCorp LLC, College Station, Texas, USA) to conduct the statistical analyses.

5 RESULTS

5.1 Patient characteristics and clinical outcomes

Study I was a *post hoc* analysis of the original FINNAKI study of 2901 patients. After excluding 857 patients, there were 2044 patients in Study I. 1330/2044 patients (65%) had baseline Cr data with a median value of 76 [62–92] μmol/l. For those 714 (35%) without baseline Cr, we used MDRD back-calculation. The baseline characteristics and the admission diagnosis groups are shown in Table 6. 1722/2044 admissions (85%) were acute. 1526/2044 (75%) patients were mechanically ventilated. The median ICU length of stay was 3 days (IQR 2–6 days); 657 patients (32%) were discharged and 125 (6%) were deceased before 5 days. 201/2044 (9.8%) patients were treated with RRT during the first 5 ICU days. Overall 90-day mortality was 22%.

Of the 182 sepsis patients in Study II, 97 (53%) had severe sepsis by the ACCP/SCCM criteria—that is, had at least one sepsis-associated OD—at the time of admission to intensive care. 85/182 (47%) patients fulfilled the criteria for severe sepsis later during the admission day or the day thereafter. 92/182 (51%) patients were diagnosed with AKI by the KDIGO criteria during the first 5 ICU days. Of the patients with AKI, 50/92 (54%) fulfilled AKI criteria on admission day, 35/92 (38%) on the following day, and 7/92 (7.6%) later during the 5-day observation period. The time of AKI diagnosis in relation to ICU admission is shown in Figure 5. AKI severity was distributed as follows: 37/92 (40%) had KDIGO stage 1, 19/92 (21%) stage 2, and 36/92 (39%) stage 3 AKI. Of all 182 patients, 26 (14%) were treated with RRT during the 5-day observation period. Baseline patient characteristics and admission diagnosis groups are shown in Table 6. When AKI and non-AKI groups were compared, history of hypertension was more frequent among patients with AKI than in those without. Additionally, septic shock was more common among patients diagnosed with AKI and they also had a higher maximum SOFA score (renal points subtracted) than patients without AKI. In logistic regression analysis of 11 baseline variables, only diabetes, systolic heart failure and maximum SOFA score without renal points were associated with AKI.

In Study II, 167 of 182 patients had a recorded time label for the first OD and were included in Study III. 166/167 (99%) patients fulfilled the latest Sepsis-3 criteria for sepsis. The onset of the first OD was later than 1 h after admission (indicating early ICU admission) in 38/167 patients (23%), ±1 h from admission in 74/167 (44%), and earlier than 1 h before admission (indicating late ICU admission) in 55/167 patients (33%). The median time between the

first OD and ICU admission was 0.0 h. The time of OD onset in relation to ICU admission is shown in Figure 6. The 3 groups, stratified according to the time of onset of the first OD, using cut points of ±1 h from admission, were comparable regarding comorbidities and disease severity, as shown in Table 7.

484 patients from the previously conducted FINNAKI NGAL -study fulfilled the Sepsis-3 definition during the first 3 ICU days and were included in Study IV. Table 6 presents characteristics and admission diagnosis groups of the included 484 critically ill sepsis patients. 217/484 (44.8%) patients developed AKI during the 3-day follow-up; 115/217 patients (53%) fulfilled AKI criteria on day 1 (admission day), 87/217 (40%) on day 2, and 15/217 (7%) on day 3. Of 46 patients treated with RRT during the first 3 ICU days, 20 (43%) commenced RRT on the first ICU day, 19 (41%) on day 2, and 7 (15%) on day 3. For uNGAL analyses, we used the first available urine sample, which was the 0 h sample in 460/484 patients (95%). 90-day mortality was 36.4% (95% CI 30.0–43.2%) among patients with AKI and 21.3% (95% CI 16.6–26.8%) in those without.

Table 6 Baseline patient characteristics in the included studies.

	Study I	**Study II** (includes Study III patients)	**Study III**	**Study IV**
Study population	ICU admissions with LOS ≥24 h	Severe sepsis (ACCP/SCCM criteria)	Severe sepsis (ACCP/SCCM criteria) and recorded onset of 1st OD	Sepsis (Sepsis-3 criteria)
Number of patients	2044	182	167	484
Age, median [IQR]	64 [53–74]	62 [52–72]	62 [53–72]	65 [54–75]
Male gender, n/data available (%)	1331/2044 (65)	115/182 (63)	108/167 (65)	310/484 (64)
Co-morbidities, n/data available (%)				
Hypertension	999/2031 (49)	87/180 (48)	77/165 (47)	255/484 (53)
Diabetes	444/2044 (22)	39/182 (21)	34/167 (20)	117/484 (24)
Atherosclerosis	276/2012 (14)	17/180 (9)	17/165 (10)	64/484 (13)
Systolic heart failure	250/2028 (12)	13/179 (7)	12/165 (7)	64/484 (13)
Chronic kidney disease	137/2034 (7)	0/182 (0)*	0/167 (0)*	35/484 (7)
Admission type, n/data available (%)				
Emergency	1722/2018 (85)	179/182 (98)	164/167 (98)	465/482 (96)
Operative	799/2043 (39)	41/181 (23)	36/166 (22)	123/484 (25)
APACHE dx groups, n/data available (%)				
Cardiovascular, operative	390/2043 (19)	2/182 (1)	2/167 (1)	23/484 (5)
Cardiovascular, non-operative	277/2043 (14)	15/182 (8)	12/167 (7)	65/484 (13)
Respiratory tract, non-operative	253/2043 (12)	58/182 (32)	58/167 (35)	109/484 (23)
Gastrointestinal tract, operative	187/2043 (9)	31/182 (17)	27/167 (16)	63/484 (13)
Neurological, non-operative	182/2043 (9)	11/182 (6)	10/167 (6)	26/484 (5)
Gastrointestinal tract, non-operative	128/2043 (6)	14/182 (8)	12/167 (7)	36/484 (7)
Metabolic	128/2043 (6)	8/182 (4)	5/167 (3)	23/484 (5)
Sepsis (not urosepsis)	120/2043 (6)	21/182 (12)	21/167 (13)	64/484 (13)
Neurological, operative	100/2043 (5)	4/182 (2)	4/167 (2)	10/484 (2)
Other	278/2043 (14)	18/182 (10)	16/167 (10)	75/484 (15)
Disease severity				
SAPS II, median [IQR]	37 [29–50]	43 [35–52]	43 [35–52]	40 [32–53]
SOFA score day 1, median [IQR]	7 [5–10]	8 [6–11]	8 [6–11]	8 [5–10]
Norepinephrine during first 24 h in ICU, n/data available (%)	1368/2044 (67)	120/182 (66)	118/167 (71)	321/484 (66)
ICU LOS, days, median [IQR]	3.1 [1.9–5.9]	4.6 [2.7–8.0]	4.5 [2.7–8.1]	4.0 [2.2–7.6]

*patients with chronic kidney disease were excluded

ACCP/SCCM, American College of Chest Physicians/Society of Critical Care Medicine; APACHE, acute physiology and chronic health evaluation; ICU, intensive care unit; IQR, interquartile range; LOS, length of stay; OD, organ dysfunction; SAPS, simplified acute physiology score; SOFA, sequential organ failure assessment

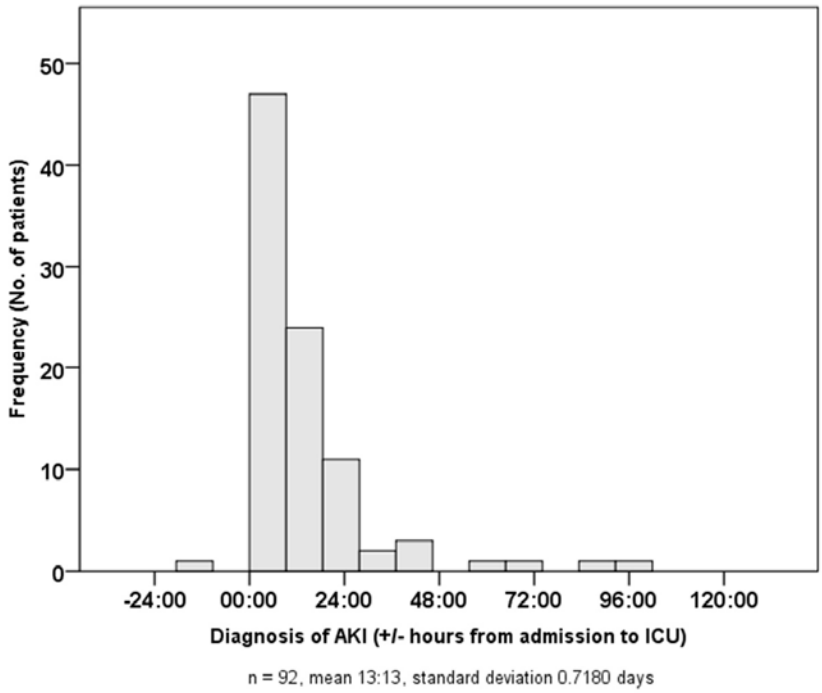


Figure 5 Time of diagnosis of acute kidney injury (AKI) in relation to intensive care unit admission in Study II. Figure by the author.

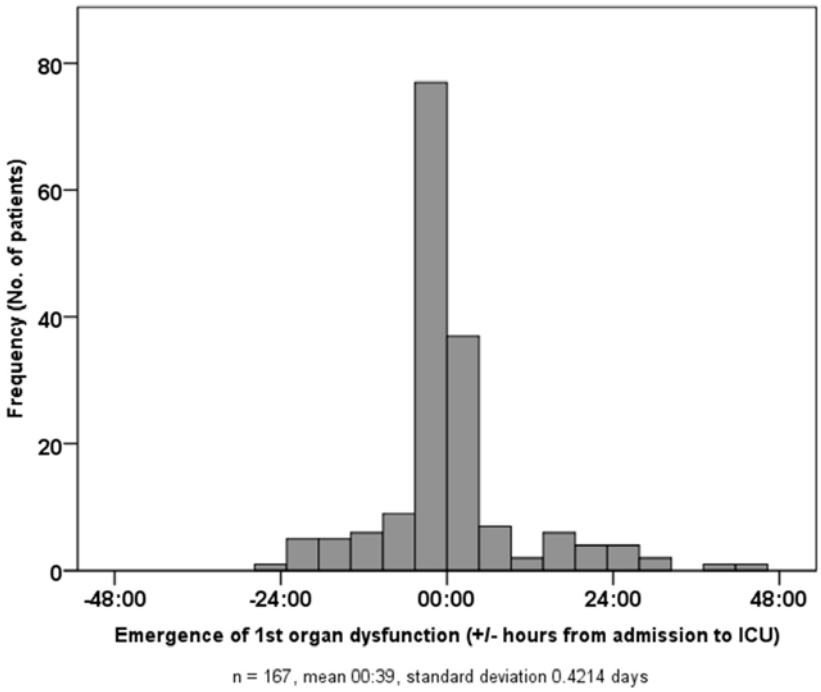


Figure 6 Time of onset of the first organ dysfunction in relation to intensive care unit admission in Study III. Figure by the author.

Table 7 Comparison of comorbidities and disease severity in the three groups with different sampling time in Study III, modified from table originally appearing in Innate Immunity 2021;27(2):192-200.

	Data available	Before OD (n=38)	At OD (n=74)	After OD (n=55)	p
Time from ICU admission to first documented OD, h, median (range)	167	12.3 (1.0–45.6)	0 (-0.9–0.9)[a]	-4.1 (-23.8–-1.2)[a]	
Age, median [IQR]	167	60 [50–71]	63 [56–73]	61 [52–72]	0.310
Male gender, n (%)	167	24 (63)	46 (62)	38 (69)	0.700
Co-morbidities, n (%)					
Hypertension	165	15 (40)	31 (42)	31 (56)	0.238
Diabetes with oral or insulin medication	167	8 (21)	12 (16)	14 (26)	0.433
Atherosclerosis	165	4 (11)	7 (10)	6 (11)	0.853
Systolic heart failure	165	3 (8)	7 (10)	2 (4)	0.451
Admission type and status					
Emergency, n (%)	167	38 (100)	72 (97)	54 (98)	0.797
Operative, n (%)	166	13 (34)	6 (8)	17 (31)	0.001
SAPS II, median [IQR]	167	41 [32–51]	44 [35–56]	44 [36–50]	0.459
Sepsis severity and organ dysfunction					
Septic shock during first 5 ICU days, n (%)	167	26 (68)	49 (66)	43 (78)	0.317
Lactate, mmol/l (first in ICU, median [IQR])	134	1.5 [1.1–2.1]	1.6 [1.0–3.7]	1.6 [1.0–4.0]	0.612
Highest SOFA, median [IQR]	167	9 [8–12]	9 [7–11]	10 [7–12]	0.342
AKI, n (%)	167	19 (50)	34 (46)	32 (58)	0.386
RRT for AKI, n (%)	167	4 (11)	10 (14)	9 (16)	0.722
ICU LOS, days, median [IQR]	167	4.6 [2.9–8.3]	4.1 [2.6–8.0]	5.0 [2.7–8.2]	0.856

"Before OD", patients who developed their first organ dysfunction > 1 h after ICU admission; "At OD", patients presenting their first organ dysfunction ± 1 h from admission; "After OD", patients who had organ dysfunction > 1 h before ICU admission.

[a]A negative value indicates that organ dysfunction has emerged before admission.

APACHE, acute physiology and chronic health evaluation; AKI, acute kidney injury; ICU, intensive care unit; IQR, interquartile range; LOS, length of stay; OD, organ dysfunction; RRT, renal replacement therapy; SAPS, simplified acute physiology score; SOFA, sequential organ failure assessment

5.2 Influence of adjusting Cr values for fluid balance (I)

In Study I, we assessed 7279 plasma Cr values of 2044 ICU patients, with the median being 4 [IQR 2–5] measurements per patient. Highest Cr was measured on day 2 [median; IQR 2–3]. The median fluid balance used to calculate the highest adjusted Cr value for each patient was 17 ml (-167–616 ml). The mean difference between all adjusted and unadjusted Cr values was 5 µmol/l (±15 µmol/l) (Figure 7).

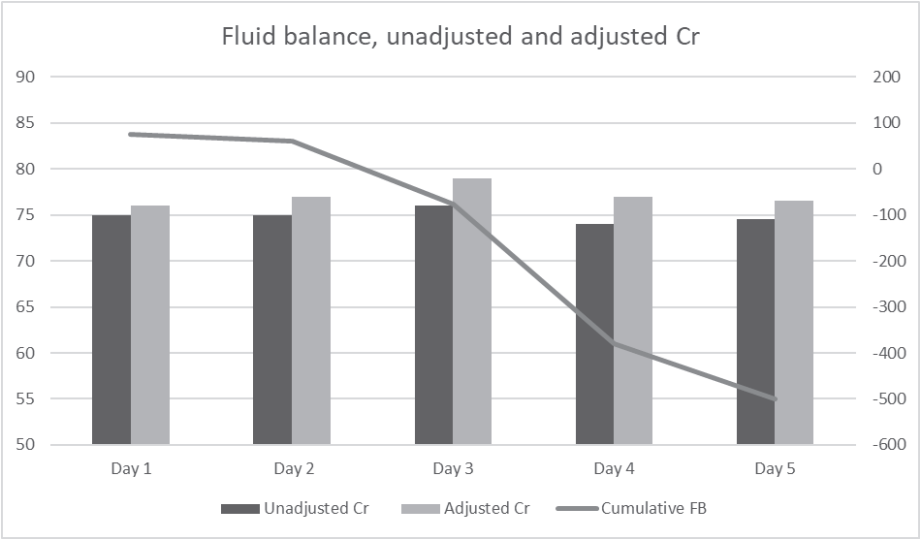


Figure 7 Mean unadjusted versus adjusted creatinine values (µmol/; y-axis, left) and median fluid balance (ml; y-axis, right) during the first 5 days of intensive care in Study I. Number of observations per day: Day 1: 1536; Day 2: 1905; Day 3: 1402; Day 4: 1018; Day 5: 760. Cr, creatinine; FB, fluid balance.

Figure originally from Törnblom et al. Acta Anaesthesiologica Scandinavica 2021;65:1079–1086 with permission from Wiley (Creative Commons Attribution-NonCommercial License).

5.2.1 Incidence of AKI (I)

Using unadjusted Cr, 616/2044 (30%; 95% CI 28–32%) patients were diagnosed with AKI. Adjusting Cr for fluid balance yielded 38 additional AKI diagnoses resulting in an incidence of 31% (95% CI 29–34%). The absolute difference between these incidences was 2% (95% CI 1–3%). 15 patients had AKI based on unadjusted Cr but not when adjusted Cr was used. 53 patients were diagnosed with AKI based on adjusted but not on unadjusted Cr. Table 8 shows cross tabulation.

When full KDIGO criteria (including unadjusted Cr, UO and RRT) were applied, AKI incidence was 44% (95% CI 41–46%), and based on the full criteria with adjusted Cr, 45% (95% CI 42–47%). The absolute incidence difference

was 0.9% (95% CI 0.3–1.6%). Thereby, using adjusted Cr with UO and RRT criteria led to AKI diagnosis in 19 additional patients (1%). The cross tabulation for the full KDIGO criteria with or without Cr adjustment is shown in Table 9. Figure 8 illustrates the highest KDIGO stages using either only Cr or full criteria with or without Cr adjustment. We detected no marked differences in AKI incidence in various subgroups when the full criteria were used (Figure 9). The number of patients changing category after adjustment was similar among those not having baseline Cr available, although their AKI incidences were lower regardless of Cr adjustment (Table 10).

Table 8 Cross tabulation of AKI in Study I based on Kidney Disease: Improving Global Outcomes (KDIGO) creatinine (Cr) criterion only (adjusted or unadjusted). Table originally from Törnblom et al. Acta Anaesthesiologica Scandinavica 2021;65:1079–1086 with permission from Wiley (Creative Commons Attribution-NonCommercial License).

	No AKI -using adjusted Cr, n (%; 95% CI)	**AKI -using adjusted Cr**, n (%; 95% CI)
No AKI based on unadjusted Cr 90-day mortality	1375 (67.3%; 65.2–69.3) 244 (17.7%; 15.7–19.8)	53 (2.6%; 1.9–3.3) 21 (39.6%; 26.5–53.9)
AKI based on unadjusted Cr 90-day mortality	15 (0.7%; 0.4–0.12) 2 (13.3%; 3.7–37.8)	601 (29.4%; 27.4–31.4) 189 (31.4%; 27.7–35.3)

Table 9 Cross tabulation of AKI in Study I based on full KDIGO criteria including adjusted or unadjusted Cr. Table originally from Törnblom et al. Acta Anaesthesiologica Scandinavica 2021;65:1079–1086 with permission from Wiley (Creative Commons Attribution-NonCommercial License).

	No AKI -adjusted Cr, n (%; 95% CI)	**AKI -adjusted Cr**, (%; 95% CI)
No AKI -unadjusted Cr 90-day mortality	1122 (54.9%; 52.7–57.1) 180 (16.0%; 13.9–18.3)	31 (1.5%; 1.0–2.2) 8 (25.8%; 11.8–44.6)
AKI -unadjusted Cr 90-day mortality	12 (0.6%; 0.3–1.0) 1 (8.3%; 4.3–35.4)	879 (43.0%; 40.8–45.2) 267 (30.4%; 27.3–33.5)

Table 10 Comparison of AKI incidence for Study I patients with and without baseline Cr. Table originally from Törnblom et al. Acta Anaesthesiologica Scandinavica 2021;65:1079–1086, supporting information, with permission from Wiley (Creative Commons Attribution-NonCommercial License).

AKI	**No baseline Cr available (n=714)**	**Baseline Cr available (n=1330)**	**P-value**
Unadjusted Cr only	183 (25.6%)	433 (32.6%)	0.001
Adjusted Cr only	189 (26.5%)	465 (35.0%)	0.001
Unadjusted full criteria	284 (39.8%)	607 (45.6%)	0.011
Adjusted full criteria	284 (39.8%)	626 (47.1%)	0.002

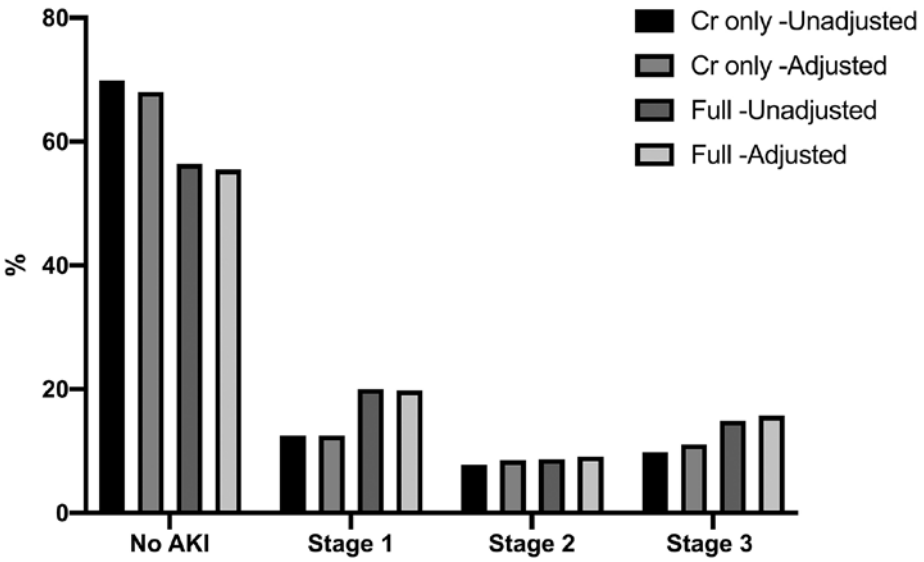


Figure 8 The highest KDIGO stages of Study I population when using either only unadjusted or adjusted Cr, or these in combination with UO and RRT criteria.

AKI, acute kidney injury; Cr, creatinine; Full, Cr and UO and RRT; KDIGO, Kidney Disease: Improving Global Outcomes.

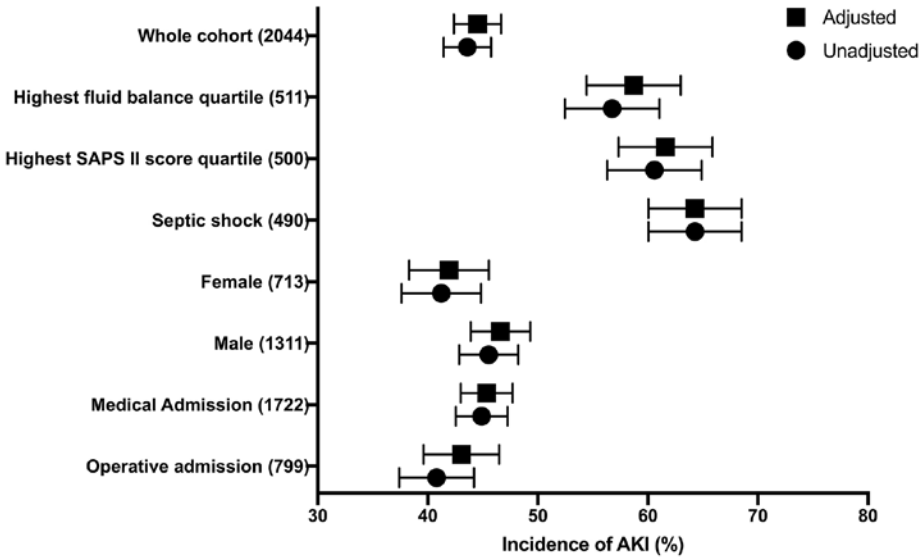


Figure 9 Incidence of AKI in Study I subgroups when using the UO, RRT, and unadjusted or adjusted Cr criteria. Error bars denote 95% confidence intervals. Numbers in parenthesis indicate the number of patients in the group. Highest fluid balance quartile >667 ml (up to 12 198 ml). Highest SAPS II score quartile >50 points (up to 102). AKI; Acute kidney injury, SAPS; Simplified Acute Physiology Score.

Figures 8-9 originally from Törnblom et al. Acta Anaesthesiologica Scandinavica 2021;65:1079–1086 with permission from Wiley (Creative Commons Attribution-NonCommercial License).

5.2.2 90-day mortality (I)

The 90-day mortality rate was 31% (95% CI 28–35%) among patients having AKI based on both unadjusted and adjusted Cr. Of those without AKI, 244 (18%) had died by 90 days. The 90-day mortality of those 53 patients who had AKI only after Cr adjustment was 40% (95% CI 26–53%), which was not significantly different compared to patients having AKI on both unadjusted and adjusted Cr criteria (absolute mortality rate difference 8%; 95% CI -7–22%). Cross tabulations with all mortality rates when using either only Cr criterion or the full criteria are shown in Tables 8 and 9, respectively.

5.3 Plasma measurements of proinflammatory cytokines and neutrophil activation markers

5.3.1 Interleukins 6 and 8 (II–III)

Sepsis patients had higher plasma IL-6 and IL-8 concentrations than the healthy controls (III; Figure 10). At 0 h and 24 h from ICU admission, patients with septic AKI had higher IL-6 and IL-8 concentrations than those without (II; Table 11). Plasma IL-6 and IL-8 at 0 h and 24 h, stratified by AKI severity, are shown in Figure 11 (II). The AUROCs of IL-6 and IL-8 for septic AKI were 0.644 (95% CI 0.562–0.725) and 0.710 (95% CI 0.634–0.785), respectively. Using "severe" (KDIGO 2–3) AKI as an outcome did not improve the AUROCs (II).

Those sepsis patients with admission plasma samples drawn >1 h before developing their first OD (indicating an early disease phase) had lower IL-6 and IL-8 concentrations than those whose sample was obtained simultaneously with the first OD emergence (±1 h), and patients with sample taken >1 h after the first OD (indicating a late disease phase) had the highest IL-6 and IL-8 levels (III, Figure 10). Both IL-6 and IL-8 levels dropped significantly during the first 24 h following the onset of the first OD (III, Figure 12).

Before Study II, we conducted a pilot study of 32 severe sepsis patients with OD onset ≤36 h before ICU admission. In this unpublished pilot cohort, plasma IL-6 concentrations 0, 12, 24, and 48 h after admission followed a similar declining pattern (Figure 13).

5.3.2 Activin A (II)

At 0 h and 24 h from ICU admission, patients with septic AKI had higher activin A concentrations than those without (Table 11). The concentrations followed AKI severity: patients with KDIGO stage 3 AKI had significantly higher activin A levels than KDIGO stage 1 patients (Figure 11). The AUROC of activin A for septic AKI was 0.706 (IQR 0.630–0.782). Using "severe" (KDIGO 2–3) AKI as an outcome did not improve the AUROC (II).

5.3.3 MPO (II–III)

Sepsis patients had higher plasma MPO concentrations than the healthy controls (III, Figure 10), but admission plasma MPO concentrations did not differ significantly between patients with and without septic AKI (II, Table 11). At 24 h from ICU admission, patients with septic AKI had higher MPO concentrations than those without (II, Table 11).

Plasma MPO concentrations did not differ between those sepsis patients whose sample was taken early, before the first OD, and those with sample taken later, after the first OD (Figure 10). Compared with healthy controls, sepsis patients already had elevated MPO before OD onset, and MPO remained stably high for at least 24 h following the first OD emergence (III, Figures 10 and 12).

5.3.4 HBP (III)

Sepsis patients had higher plasma HBP concentrations than the healthy controls (III, Figure 10). Plasma HBP concentrations did not differ between those sepsis patients, whose sample was taken early, before the first OD, and those with sample taken later, after the first OD (Figure 10). Compared with healthy controls, sepsis patients already had elevated HBP before OD onset, and HBP remained stably high for at least 24 h following the first OD emergence (III, Figures 10 and 12).

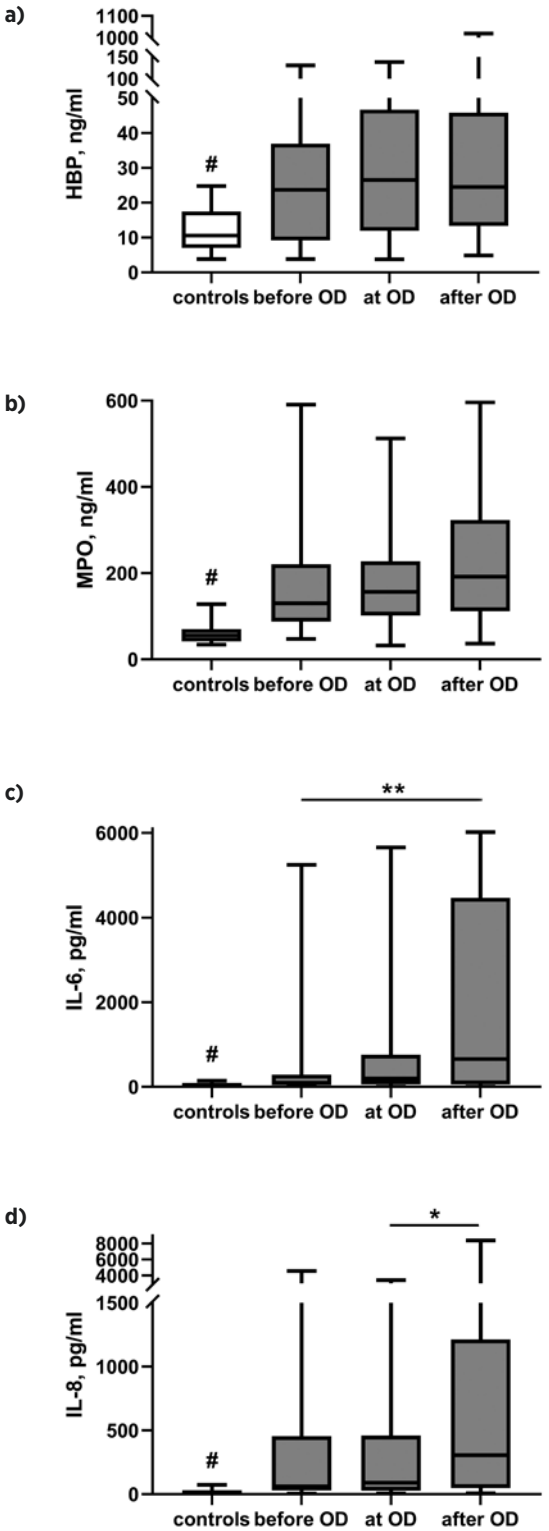


Figure 10 Plasma inflammatory biomarkers
a) HBP,
b) MPO,
c) IL-6, and
d) IL-8 in controls and in the three groups with different sampling times in relation to the onset of organ dysfunction (OD).

# p <0.01, controls vs. "before OD"/ "at OD"/ "after OD";
* p <0.05 and ** p <0.01, among sepsis patients.
Study III, figure appearing originally in Innate Immunity 2021;27(2):192-200.

Table 11 Plasma and urine biomarkers according to the presence or absence of AKI in Study II, originally from Törnblom et al. Acta Anaesthesiologica Scandinavica 2019;63:1390-1397. Reproduced with permission from Wiley.

		All	No AKI (n=90)	AKI (n=92)	AKI vs. no AKI
Activin A (pg/ml)	**Plasma 0 h**	661 [368–1287]	469 [285–862]	845 [554–1895]	P <0.001
	Plasma 24 h	563 [389–1060]***	470 [301–827]	795 [433–1363]**	P <0.001
	Urine 0 h	7.1 [0.0–41.1]	4.0 [0.0–33.0]	9.7 [1.4–42.6]	P =0.064
IL-8 (pg/ml)	**Plasma 0 h**	101 [30–673]	50 [19–164]	240 [60–971]	P <0.001
	Plasma 24 h	47 [20–120]***	32 [13–74]***	79 [31–219]***	P <0.001
	Urine 0 h	23.5 [6.9–95.5]	9.5 [2.7–28.7]	50.4 [19.8–145.3]	P <0.001
IL-6 (pg/ml)	**Plasma 0 h**	203 [53–947]	109 [38–366]	402 [73–2148]	P =0.001
	Plasma 24 h	101 [31–278]***	52 [0–143]***	182 [67–696]***	P <0.001
	Urine 0 h	48.6 [20.6–80.7]	37.8 [11.4–67.2]	67.7 [28.7–147.9]	P <0.001
MPO (ng/ml)	**Plasma 0 h**	151 [100–248]	144 [88–215]	169 [111–300]	P =0.059
	Plasma 24 h	167 [110–261]*	132 [103–211]	207 [136–323]**	P <0.001
	Urine 0 h	3.9 [1.0–10.5]	1.9 [0.4–6.9]	7.7 [1.5–12.6]	P <0.001
B-Leukocytes min (E9/l)		10.3 [5.5–15.6]	9.2 [5.4–13.5]	11.4 [6.1–16.1]	P =0.19
P-CRP max (mg/l)		163 [61–259]	122 [49–256]	173 [102–267]	P =0.11

Data are expressed as median [IQR]. Urine was available in 118 patients. Minimum leukocyte count and maximum P-CRP values ≤24 h prior to ICU admission were available in 160 and 172 patients, respectively.

*p <0.05; **p <0.01; ***p <0.001, 24 vs. 0 h.

AKI, acute kidney injury; ICU, intensive care unit; IL-8, interleukin 8; IL-6, interleukin 6; IQR, interquartile range; MPO, myeloperoxidase; P-CRP, plasma C-reactive protein

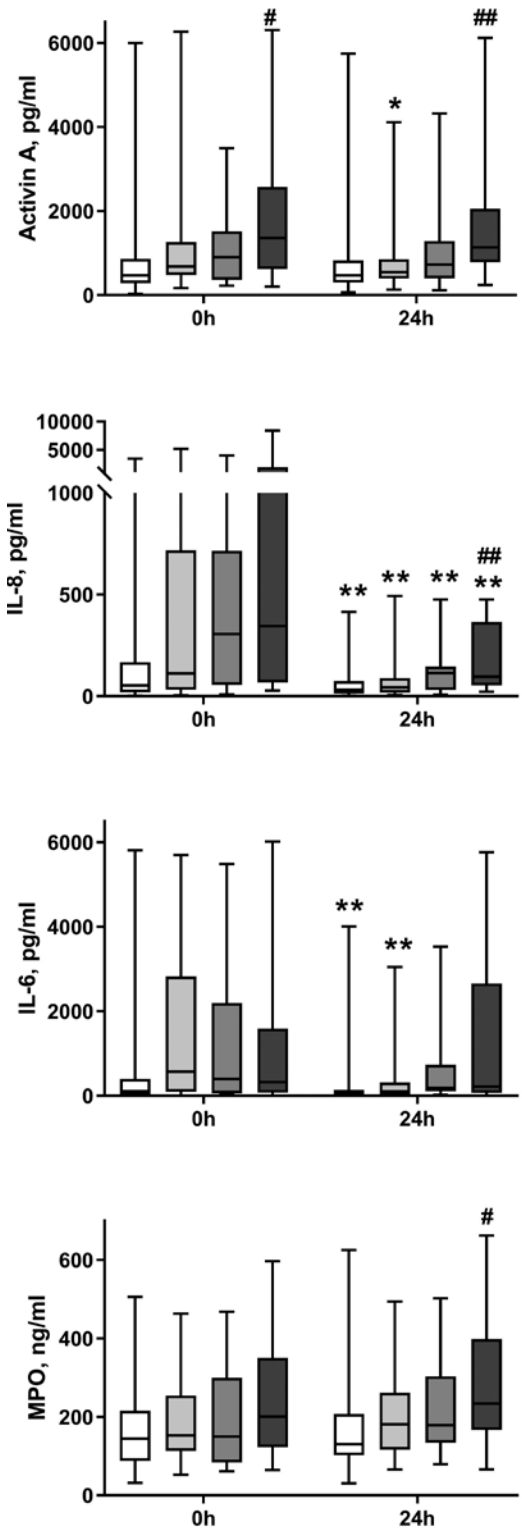


Figure 11 Plasma activin A, IL-8, IL-6, and MPO upon ICU admission (0 h) and 24 h thereafter (24 h) in Study II patients without AKI (white bars), and with KDIGO stage 1 (the lightest gray bars), stage 2 (gray bars), and stage 3 AKI (the darkest gray bars).

* p <0.01 and ** p <0.005, 0 h vs. 24 h in the Mann-Whitney test with the Bonferroni corrected significance level of p <0.0125;

# p <0.05 and ## p <0.01, KDIGO stage 1 vs. KDIGO stage 3 in the Kruskal–Wallis test with pairwise comparison as the post hoc test.

Figure originally from Törnblom et al. Acta Anaesthesiologica Scandinavica 2019;63:1390-1397. Reproduced with permission from Wiley.

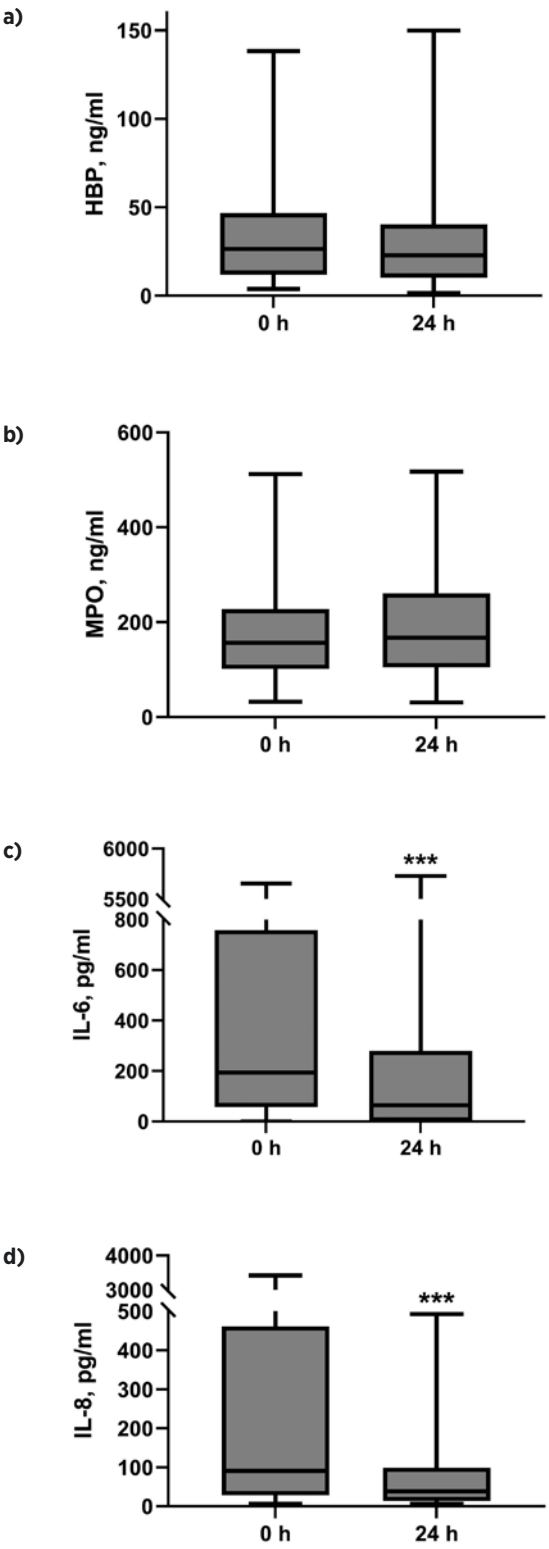


Figure 12 Plasma inflammatory biomarkers
a) HBP,
b) MPO,
c) IL-6, and
d) IL-8 in Study III subgroup of patients with onset of organ dysfunction ±1 h from intensive care unit admission, at 0 h (admission) and 24 h later (24 h; n =74), appearing originally in Innate Immunity 2021;27(2):192-200.

*** p <0.001, 0 h vs. 24 h

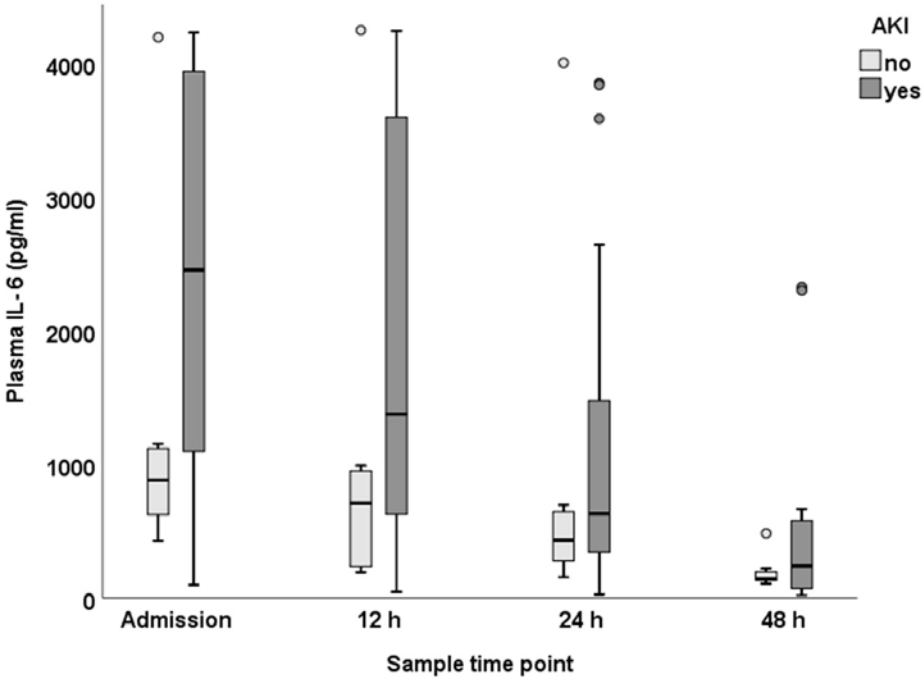


Figure 13 Unpublished data from the pilot study of 32 sepsis patients, stratified by AKI status, showing a decline in plasma IL-6 during the first 48 h of ICU stay. Small circles mark outliers. Figure by the author.

5.4 Urine measurements of proinflammatory cytokines and neutrophil activation markers

5.4.1 Interleukins 6 and 8, activin A, and MPO (II)

In Study II, 118/182 severe sepsis patients had admission urine samples available for measurements. The groups with and without urine samples did not differ regarding comorbidities and disease severity, but those without urine samples were slightly younger: 59 (IQR 47.25–67.75) vs 63 (IQR 55–73) years; $p<0.05$. Urine IL-6, IL-8 and activin A correlated only weakly with the corresponding plasma measurements ($r=0.335–0.415$; all $p<0.01$), while urine and plasma MPO did not correlate at all. AKI patients had higher urine IL-6, IL-8 and MPO concentrations than those without AKI, but activin A did not differ between these groups (Table 11). There was a positive correlation between urine IL-8 and urine MPO ($r=0.627$, $p<0.001$). Of the four measured biomarkers, only IL-8 concentrations increased with AKI severity, being significantly higher in KDIGO stage 3 patients than in KDIGO 1 patients (Figure 14). The AUROCs of IL-6, IL-8 and MPO for AKI were moderate and comparable to the AUROCs of the corresponding plasma measurements.

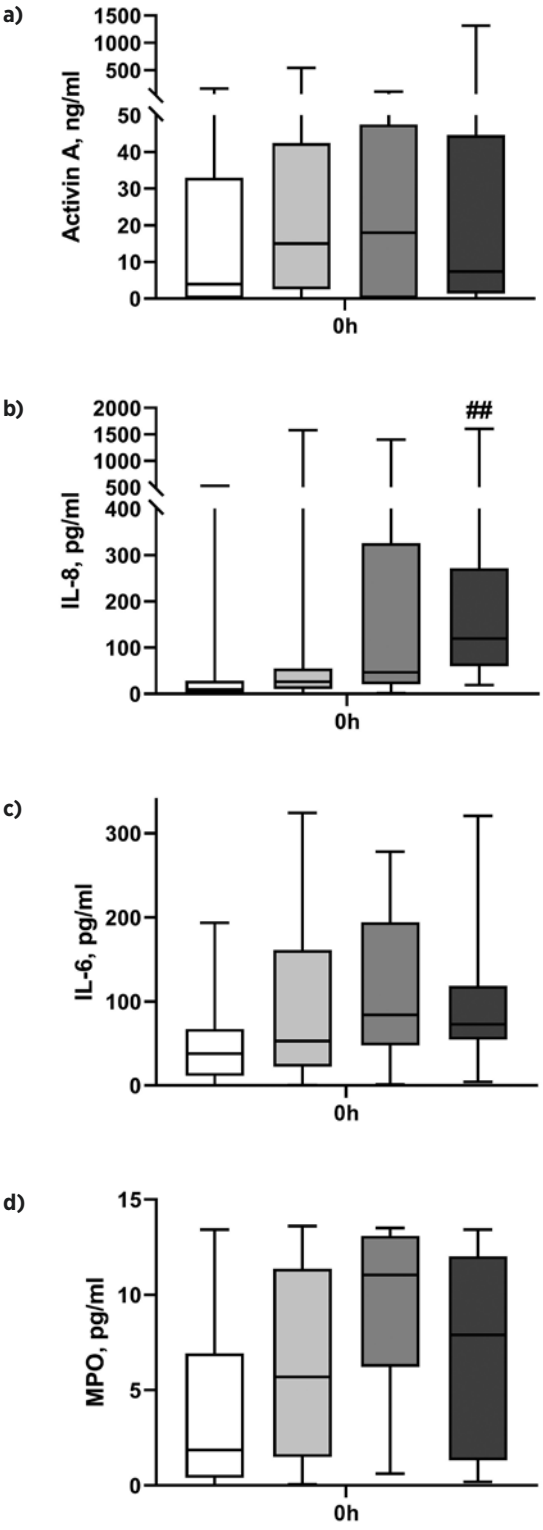


Figure 14 Urine inflammatory biomarkers
a) activin A,
b) IL-8,
c) IL-6, and
d) MPO upon admission to intensive care in Study II patients without AKI (white bars), with KDIGO stage 1 AKI (the lightest gray bars), stage 2 AKI (gray bars), and stage 3 AKI (the darkest gray bars).

p <0.01, KDIGO stage 1 vs. stage 3 AKI in the Kruskal–Wallis test with pairwise comparison as the post hoc test. Urine was available in 118/182 sepsis patients.

Figure originally from Törnblom et al. Acta Anaesthesiologica Scandinavica 2019;63:1390-1397. Reproduced with permission from Wiley.

5.4.2 Usefulness of uNGAL to predict AKI, RRT, and 90-day mortality in sepsis (IV)

Table 12 shows the associations of variables available at the time of ICU admission with the four studied outcomes (AKI, "severe" AKI, RRT, and 90-day mortality). Variables with the strongest associations, marked with asterisks, were entered in the multivariable logistic regression analyses to construct the clinical risk models. The AUROCs for uNGAL alone to predict the four studied outcomes—AKI, "severe" (KDIGO stage 2–3) AKI, RRT, and death—were 0.690 (95% CI 0.647–0.731), 0.728 (95% CI 0.686–0.767), 0.769 (95% CI 0.729–0.806), and 0.600 (95% CI 0.555–0.644), respectively. The corresponding AUROCs for the clinical risk models were between 0.717 and 0.797, shown with 95% CIs in Table 13. We found statistically significant model improvement when uNGAL was added to the clinical risk models to predict AKI, severe AKI, and RRT (Table 13, Figure 15a–c). The 90-day mortality prediction did not improve (Table 13, Figure 15d). Despite observed model improvement predicting AKI, severe AKI, and RRT, the net benefits in the range of the predefined threshold probabilities (0.3, 0.2, and 0.1, respectively; Figure 16a–c) were modest: 2.5% (95% CI 0.2–4.6%) for AKI, 1.4% (95% CI 0.4–4.1%) for severe AKI, and 1.4% (95% CI 0.1–2.8%) for RRT. The corresponding test trade-offs were 40 (AKI), 71 (severe AKI), and 74 (RRT), meaning that 40 to 74 patients would have to be tested for one extra true-positive.

Table 12 Associations of variables explored in the univariable models with outcomes in Study IV. Modified from table in Törnblom et al. Annals of Intensive Care 2020;10:51, supplementary information, with permission from SpringerOpen (Creative Commons Attribution 4.0 International License).

	Data available (of 484)	**Two-sided p-values for outcomes**			
		AKI (KDIGO 1–3, n=217)	**Severe AKI (KDIGO 2–3, n=134)**	**RRT (n=46)**	**Death by day 90 (n=136)**
Age	484	0.022*	0.450*	0.160*	<0.001*
Gender (male)	484	0.636*	0.180*	0.339	0.119*
Diabetes	484	0.663*	0.016	0.113*	0.204*
CKD	484	0.016*	0.023*	0.003*	0.009*
COPD	476	0.221*	0.241*	0.801	0.680
Liver disease	478	0.596*	1.000*	1.000	<0.001*
Systolic heart failure	484	0.958*	0.404	0.269	0.025*
Hypertension	484	0.605*	0.669*	0.590	0.236*
Atherosclerosis	484	0.195*	0.715*	1.000	0.294*
ACE or ARB	477	0.295*	0.783*	1.000	0.792
NSAID	466	0.279*	0.263*	0.611	0.547*
Corticosteroids	481	0.506*	0.859*	0.856	0.001*
Pre-ICU hypovolemia	484	<0.001*	<0.001*	0.003*	0.598
Pre-ICU diuretics	484	0.040*	0.020*	0.700	0.184*
Pre-ICU colloids	484	0.109*	0.179*	0.422	0.737
Pre-ICU hypotension	484	<0.001*	<0.001*	0.013*	0.007*
Nonoperative admission	484	0.776*	0.407	0.672	0.005*
Emergency surgery	482	0.769*	0.336	0.200	0.024*
SAPS II (-age and renal points)	484	0.192*	0.491*	0.466	<0.001*
Highest lactate (day 1)	484	<0.001*	<0.001*	0.002*	<0.001*
Acute liver failure	484	0.143*	0.008*	0.163	0.002*

Variables with smallest p-values*, restricting the number of covariates to 1 per 8 dependent endpoints, were included in multivariate logistic regression analyses with corresponding endpoints.

ACE, angiotensin convertase enzyme-inhibitor (permanent medication); ARB, angiotensin II receptor blocker (permanent medication); CKD, chronic kidney disease; colloids, starch or gelatin; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; NSAID, non-steroid anti-inflammatory drug (permanent medication); SAPS II, Simplified Acute Physiology Score

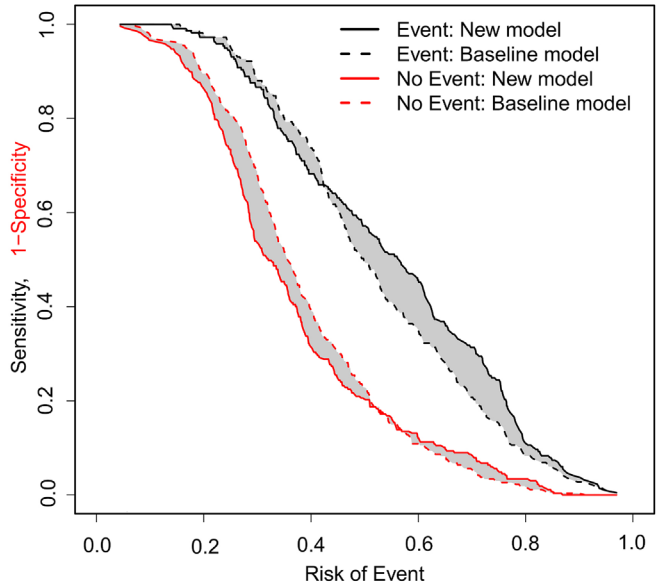
Table 13 Model improvement with uNGAL added to the clinical risk models for the endpoints (IV). Modified from table originally appearing in Annals of Intensive Care 2020;10:51 (SpringerOpen, Creative Commons Attribution 4.0 International License).

		AKI	Severe AKI (KDIGO 2–3)	RRT	90-day mortality
Goodness of fit[a]					
	clinical risk model	0.406	0.400	0.973	0.365
	new model incl. uNGAL	0.395	0.338	0.749	0.990
Events (n)		217	134	46	136
Nonevents (n)		267	350	438	348
AUROC	uNGAL alone	0.690 (0.647–0.731)	0.728 (0.686–0.767)	0.769 (0.729–0.806)	0.600 (0.555–0.644)
	clinical risk model	0.717 (0.670–0.764)	0.759 (0.710–0.809)	0.724 (0.643–0.805)	0.797 (0.754–0.840)
	new risk model (clinical + uNGAL)	0.749 (0.704–0.794)	0.799 (0.755–0.843)	0.824 (0.761–0.886)	0.804 (0.762–0.846)
	difference, *p* (clinical vs. new model)	0.017	0.011	0.005	0.27
Category-free NRI (%)	$cfNRI_{events}$	1.4 (-12.0–14.8)	22.4 (6.1–38.7)	47.8 (21.9–73.7)	-4.4 (-21.1–12.2)
	$cfNRI_{nonevents}$	48.7 (38.3–59.1)	49.1 (40.0–58.3)	46.6 (38.5–54.6)	34.5 (24.7–44.3)
	cfNRI	50.1 (33.0–67.1)	71.5 (52.9–90.2)	94.4 (67.2–121.7)	30.1 (10.7–49.4)
IDI and summary statistics	IDI_{events}	0.0248 (0.0099–0.0398)	0.0398 (0.0185–0.0610)	0.0615 (0.0361–0.0868)	0.0115 (-0.0003–0.0233)
	$IDI_{nonevents}$	0.0202 (0.0092–0.0312)	0.0152 (0.0049–0.0256)	0.0065 (-0.0007–0.0136)	0.0045 (-0.0008–0.0099)
	IDI	0.0450 (0.0264–0.0637)	0.0550 (0.0317–0.0782)	0.0679 (0.0413–0.0945)	0.0160 (0.0032–0.0289)
	IS_{old}	0.5263 (0.5006–0.5519)	0.4080 (0.3711–0.4449)	0.1947 (0.1344–0.2550)	0.4509 (0.4112–0.4905)
	IS_{new}	0.5511 (0.5226–0.5796)	0.4476 (0.4083–0.4870)	0.2557 (0.1917–0.3197)	0.4623 (0.4214–0.5032)
	IP_{old}	0.3849 (0.3651–0.4048)	0.2267 (0.2111–0.2422)	0.0846 (0.0781–0.0911)	0.2146 (0.1968–0.2325)
	IP_{new}	0.3648 (0.3425–0.3871)	0.2115 (0.1937–0.2293)	0.0782 (0.0693–0.0871)	0.2102 (0.1920–0.2283)

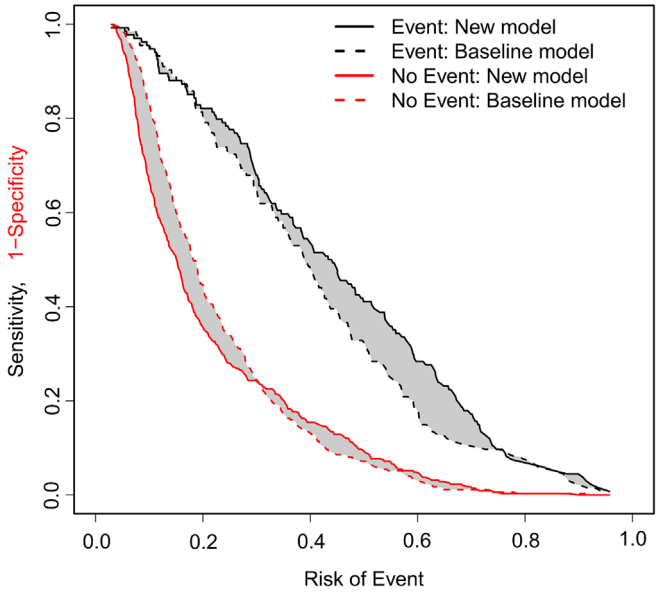
95% confidence intervals are shown in parentheses. [a]A Hosmer–Lemeshow goodness of fit was used to test calibration of the models. "New" refers to the classification model that includes the new biomarker and "old" refers to the classification model that does not.

AKI, acute kidney injury; AUROC, area under the receiver operating characteristic curve; cfNRI, category-free net reclassification improvement; IDI, integrated discrimination improvement;
$cfNRI = cfNRI_{events} + cfNRI_{nonevents}$; IS, integrated sensitivity; IP, integrated 1-specificity;
$IDI = (IS_{new} - IS_{old}) - (IP_{new} - IP_{old})$;
KDIGO, Kidney Disease: Improving Global Outcomes; RRT, renal replacement therapy; uNGAL, urine neutrophil gelatinase-associated lipocalin

a)


Event: New model
Event: Baseline model
No Event: New model
No Event: Baseline model
Sensitivity, 1−Specificity
0.0
0.2
0.4
0.6
0.8
1.0
Risk of Event

b)


Event: New model
Event: Baseline model
No Event: New model
No Event: Baseline model
Sensitivity, 1−Specificity
0.0
0.2
0.4
0.6
0.8
1.0
Risk of Event

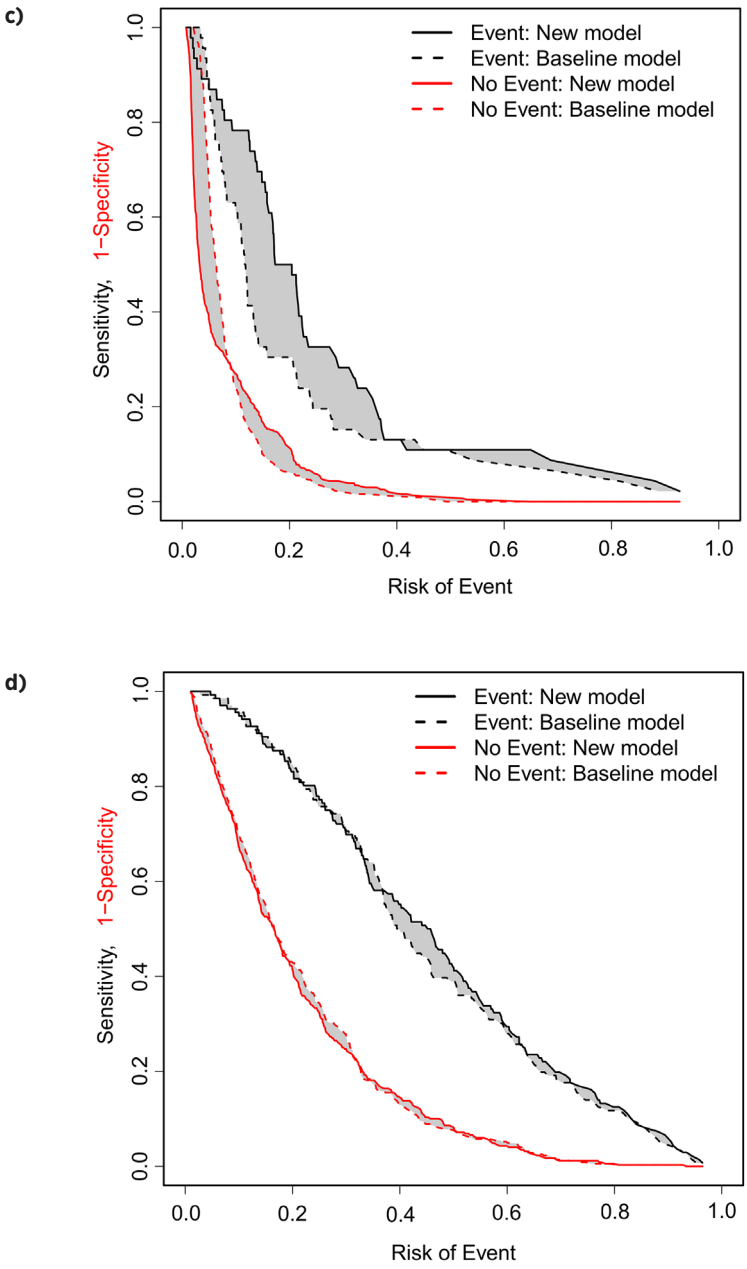
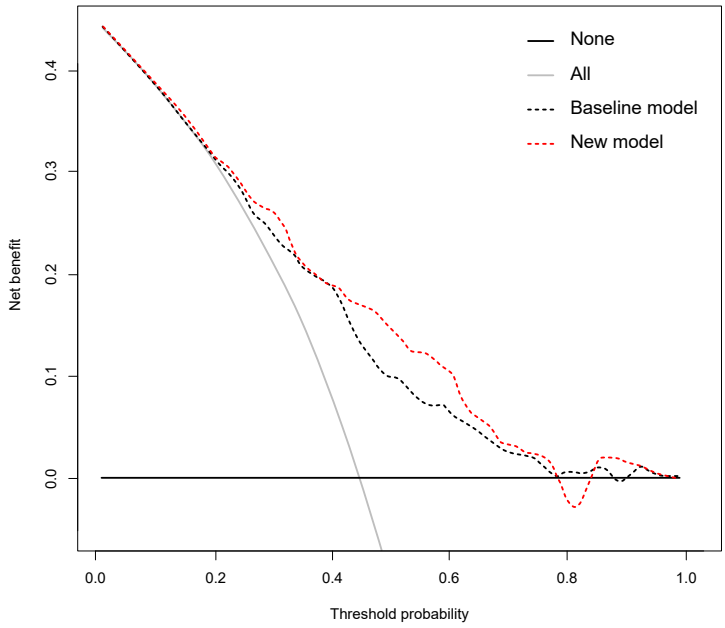


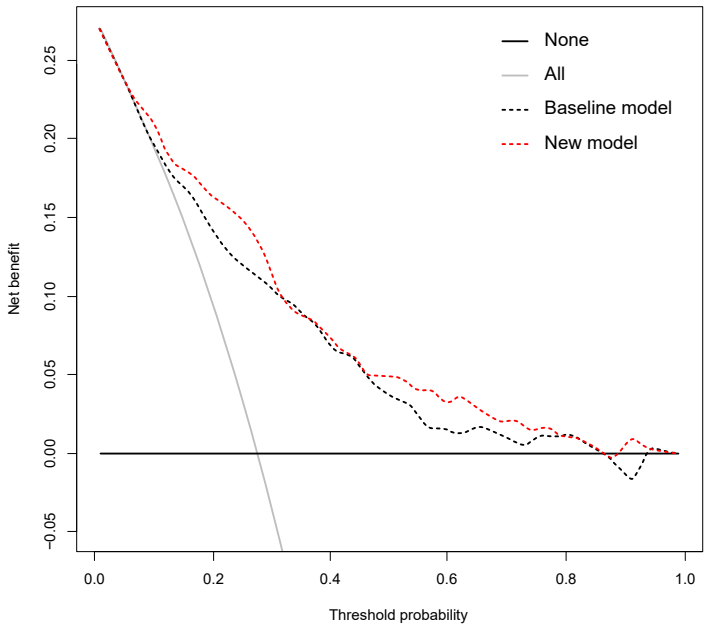
Figure 15 a-d Model enhancement in a) AKI, b) severe (KDIGO stage 2–3) AKI, c) RRT, and d) 90-day mortality presented using risk assessment plots (RAPs) for the clinical risk models (baseline model, dashed lines) and new risk models (solid lines). The gray areas between the solid and the dashed lines represent IDI_{events} (area between black lines) and $IDI_{nonevents}$ (area between red lines). (IV)

IDI, integrated discrimination improvement. Reproduced from Törnblom et al. Annals of Intensive Care 2020;10:51, with permission from SpringerOpen (Creative Commons Attribution 4.0 International License).

a)


None
All
Baseline model
New model
Net benefit
0.0
0.1
0.2
0.3
0.4
0.0
0.2
0.4
0.6
0.8
1.0
Threshold probability

b)


None
All
Baseline model
New model
Net benefit
−0.05
0.00
0.05
0.10
0.15
0.20
0.25
0.0
0.2
0.4
0.6
0.8
1.0
Threshold probability

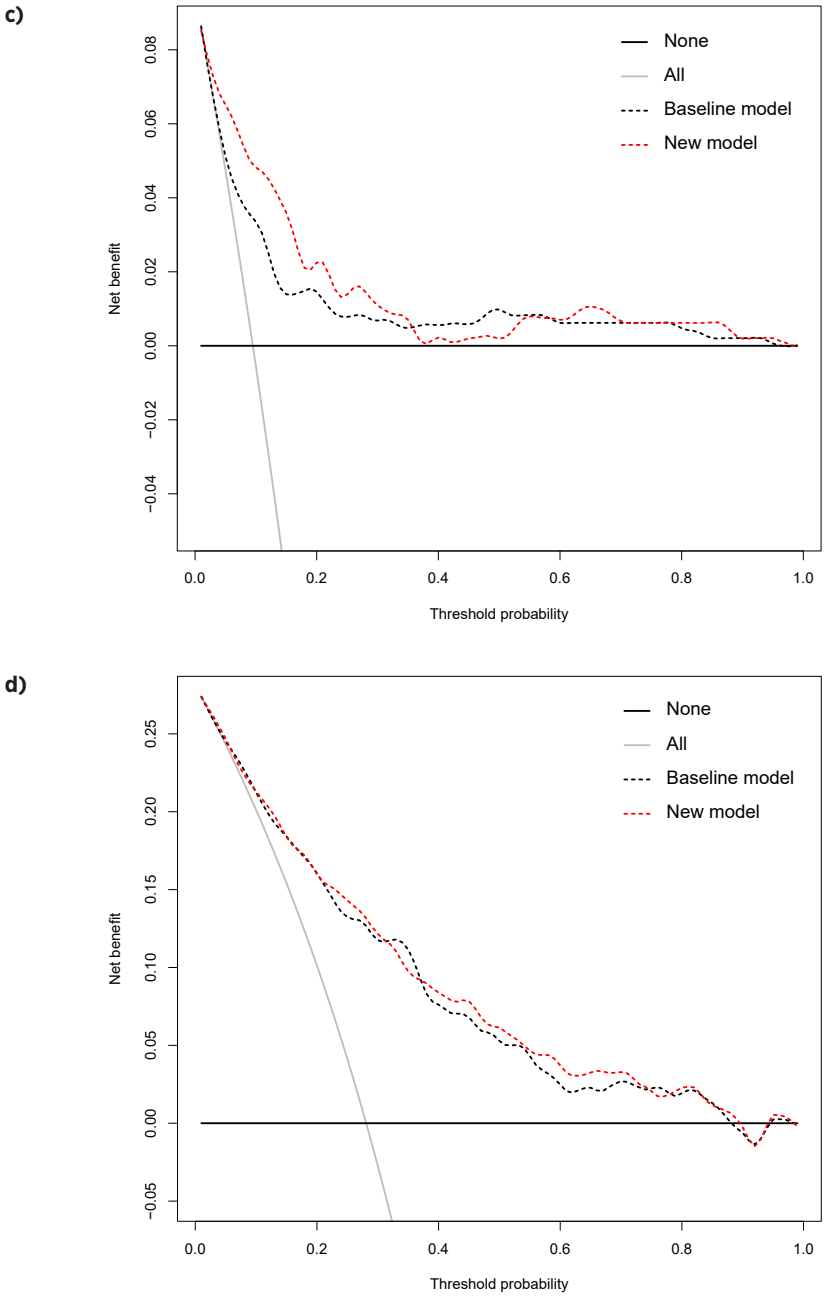


Figure 16 a-d Decision curve analysis (DCA) for the clinical risk model (baseline model, dashed black line) and clinical model with urine neutrophil gelatinase-associated lipocalin (NGAL) (new model, dashed red line) to predict a) AKI, b) severe (KDIGO stage 2–3) AKI, c) RRT, and d) 90-day mortality. Black solid line: Assume no patient has the outcome. Gray solid line: Assume all patients have the outcome. A net benefit at a certain threshold probability is present for the new model when its curve runs above the curve of the baseline model (IV). Reproduced from Törnblom et al. Annals of Intensive Care 2020;10:51, with permission from SpringerOpen (Creative Commons Attribution 4.0 International License).

6 DISCUSSION

AKI is a growing burden for hospitalized patients [228], which encourages a search for better biomarkers than the traditionally used Cr. Although still included in the latest AKI definition [45], Cr seems too late a marker to detect high-risk patients for trials with new pharmacotherapies [133]. Another concern is that fluid resuscitation given to ICU patients in high volumes may dilute plasma Cr concentration and further delay AKI diagnosis [57]. Even though AKI is a heterogenous syndrome [229], in intensive care patients, up to 50% of AKI is sepsis-induced [6,65,69,179]. To effectively prevent and treat AKI of the critically ill, understanding the mechanisms of septic AKI is therefore crucial. Undoubtedly, AKI biomarkers intended for clinical use should also be suitable for sepsis patients. The inflammatory nature of septic AKI has been revealed lately [16]. Unfortunately, many proposed AKI biomarkers are involved in systemic inflammation and lack specificity for AKI [132].

6.1 Effect of adjusting Cr for fluid balance on AKI incidence and on 90-day mortality (I)

The hemodynamic instability of the critically ill often requires aggressive fluid resuscitation, especially during the first hours of intensive care. Even after this initial stabilization, fluid accumulation is common in septic as well as in other ICU patients [230]. Fluid overload worsens prognosis [89,90], and may dilute plasma Cr [47], possibly biasing AKI incidence and staging [57]. Our retrospective analysis of 2044 ICU patients showed that the number of patients changing category in either direction after adjusting Cr was 68 (3%) when only Cr criteria were used and 43 (2%) when applying the full KDIGO criteria. Even in the highest fluid balance quartile, when Cr was used as the only criterion, less than 5% got reclassified. To summarize, adjusting Cr for fluid balance led to minimal AKI incidence differences and the influence was even smaller if the full KDIGO criteria were applied to our study population.

Previous studies have reported higher reclassification rates and delayed AKI diagnosis [47,58]. However, comparison is not straightforward because these studies used older and less sensitive AKI criteria and did not apply UO criteria. Additionally, in the Macedo study, the proportion of patients with pre-existing CKD was substantially higher than in ours (31% vs 7%) [47]. Most importantly, a marked decrease in median cumulative fluid balance has occurred between studies of the early 2000s, that reported positive balances of 5 to 10 liters [47,231],

and our study, conducted in 2011–2012. As fluid therapy protocols have evolved towards being more restrictive over the last ten years, the need for adjusting Cr for fluid balance has probably diminished.

Some scholars have argued that "unrecognized" AKI is associated with increased risk of death. Liu and colleagues analyzed data from the 1000 Fluids and Catheters Treatment trial participants and observed higher mortality (31%) among patients whose AKI was identified only after adjusting for positive fluid balance than in patients who had AKI before but not after adjustment (11%) [58]. They defined AKI by AKIN criteria without UO [58]. Our 90-day mortality results using KDIGO Cr criteria are similar, but the low number of reclassified patients warrants caution when interpreting this finding. The observed higher cumulative fluid balance, known to be associated with mortality, in those classified as having AKI only after Cr adjustment, is a possible confounding factor. When we applied the full KDIGO criteria set, with either adjusted or non-adjusted Cr, to define AKI, we did not observe mortality difference between those changing category in opposite directions (AKI→no AKI vs. no AKI→AKI). This supports the assumption that using UO criteria better detects high-risk patients than Cr alone.

6.2 Association of inflammation and neutrophil activation with septic AKI (II)

In line with earlier reports [18,19], the current study demonstrated that plasma IL-6 is associated with septic AKI. As anticipated, we also detected higher IL-8 levels among sepsis patients with AKI than among those without. Furthermore, plasma activin A, a cytosolic cytokine released from activated neutrophils, was associated with septic AKI. These findings support the suggested link between systemic inflammation, neutrophil activation, and septic AKI. However, more intense inflammation in patients with more severe disease, accompanied by OD such as AKI, is not unexpected, and not proof of an actual renal pathophysiological mechanism. Beyond intravascular inflammation and neutrophil activation, we also found that concomitant urine IL-6, IL-8 and MPO concentrations were higher in sepsis patients with AKI than in those without. Urine interleukins and MPO may either derive from plasma via renal excretion, or, locally, from the kidneys. Interestingly, plasma and urine concentrations of each studied biomarker did not have strong correlations. On the contrary, MPO in urine was associated with septic AKI but in plasma was not and, *vice versa*, activin A in plasma was associated with septic AKI but in urine was not. A few aspects could explain this. Being located in the neutrophil cytosol, activin A can be quickly released into circulation when

neutrophils get activated [20-22,143], whereas activated neutrophils secrete MPO principally after they have extravasated in the tissues [26]. The lack of correlation of urine MPO with plasma MPO might indicate that urine MPO is not derived from plasma but from the kidney itself, possibly reflecting renal accumulation of activated neutrophils, which has already been observed in experimental AKI [115,116]. This theory is further supported by the observed positive correlation between neutrophil activation marker MPO and neutrophil chemoattractant IL-8 in urine. Because direct biopsy-based organ-level investigation of septic AKI mechanisms is risky for patients and not ethically acceptable, we were able to study neutrophil activation only indirectly.

6.3 Timing of the "cytokine storm" and systemic neutrophil activation in sepsis (III)

It has been previously shown that experimental endotoxemia, a model designed to mimic sepsis, elevates IL-6 and IL-8 concentrations of healthy volunteers within 1–2 h from LPS dose [232,233]. However, this model has been criticized for differing from clinical sepsis caused by bacterial invasion and for being unsuitable for studying septic AKI [234]. We observed a sharp IL-6 and IL-8 rise in sepsis patients' plasma near the time of OD onset in a "real life" clinical setting. The concentrations peaked during the first 24 h from OD onset but also decreased rapidly. Neutrophil activation markers HBP and MPO, on the other hand, were already over two times higher in sepsis patients than in healthy controls at a median of 12 h before the first observed OD. Furthermore, we did not detect a significant decline in HBP and MPO levels during the first 24 h following OD onset—a pattern reported previously [31]. This early and stable elevation contrasts with the short-lived peaking of IL-6 and IL-8. In other words, during the first 24 h of intensive care, sepsis patients' IL-6 and IL-8 levels varied much more than their HBP and MPO concentrations. Thus, the risk for taking a sample at a "wrong" time seems smaller for HBP and MPO. Our findings indicate that HBP and MPO might outperform IL-6 and IL-8 as biomarkers, supporting previous studies claiming that HBP could be used as a biomarker for OD and AKI [28,235] , and that MPO is associated with OD and mortality among sepsis patients [30,31].

6.4 Added value of uNGAL as a biomarker for septic AKI (IV)

Our findings suggest that uNGAL alone is not superior to our clinical risk models using routine admission data for AKI prediction in sepsis. Adding uNGAL to the clinical risk models improved them slightly, yielding point estimate AUROCs of 0.749 and 0.799 for AKI and severe AKI prediction, respectively. However, looking at DCAs and test trade-offs, testing 40 to 71 patients to gain one extra true-positive is hardly worthwhile. Based on these results, we cannot recommend using uNGAL to predict septic AKI.

The present results differ from the previous meta-analysis by Zhang and colleagues, who reported an AUROC of 0.90 for uNGAL to predict septic AKI [236]. In our cohort of 484 sepsis patients, AUROCs were substantially lower for both AKI and severe (KDIGO 2–3) AKI, and not better than the AUROCs of clinical risk models using the information available upon ICU admission. A possible explanation for this discrepancy is that almost two–thirds of Zhang's 1263 meta-analysis patients were from a single country, China, and 228 were from emergency departments, not ICUs [236]. Of note, one of the included studies, conducted in Danish ICUs, reported AUROCs comparable to ours—possibly indicating a similar patient population, setting, and clinical practice [237]. Surprisingly, the included biomarker studies presenting AUROCs do not always give the cut-offs used to constitute high- and low-risk groups, although those are obligatory to guide decision making in a clinical setting. Many biomarkers have performed well in standardized study conditions, but the clinical reality is often more complex. The varied (gradual to rapid) or even fluctuating AKI development complicates defining the optimal time for drawing the sample. The environment is far more challenging than, for instance, in cardiac ischemia presenting with chest pain that starts at an easily identifiable time point.

6.5 uNGAL as a prognostic biomarker for RRT and 90-day mortality among sepsis patients (IV)

The previous FINNAKI substudy reported that uNGAL predicted RRT with an AUROC of 0.839 [32]. As anticipated, selecting only sepsis patients lowered the point estimate AUROC for RRT prediction (AUROC 0.769). Again, the clinical risk model (AUROC 0.724) and uNGAL combined improved RRT prediction only modestly—not clinically meaningfully. However, the number of outcome events (RRT) was small, so these results must be interpreted with caution. When it comes to sepsis patients' 90-day mortality, according to our study, uNGAL offered no predictive support at all.

6.6 Study strengths, limitations, and methodological considerations

The main strength of our study is the prospective data collection of a large critically ill population from 17 Finnish ICUs [4]. Importantly, the cohort comprised nonselected adult patients with precise sepsis documentation, recorded ODs and AKI criteria, including UO. When we studied a patient sample from a larger study (II–III), we included consecutive patients to maintain generalizability of the results. We used the most recent AKI definition. At the time of patient enrollment, sepsis was defined using the ACCP/SCCM criteria [37]. In Studies III–IV, we *post hoc* applied the latest Sepsis-3 criteria [42]. We increased the reliability of our results by assessing more than one neutrophil activation marker (II–III), and by measuring neutrophil activation markers simultaneously in plasma and in urine (II). Additionally, we conducted a separate pilot study with plasma and urine samples from 32 sepsis patients to calculate sample size. At all times, the researchers performing the analyses were blinded to clinical data. All studies (I–IV) were researcher-driven without any economical or other conflicts of interests. Three out of four studies (I, III–IV) were published in an open access format to advance open science principles.

In Study IV, we conducted a thorough statistical analysis with novel statistical tools to assess the clinical usefulness of uNGAL for prediction of AKI, RRT, and 90-day mortality. We extended uNGAL assessment beyond the c-statistics commonly used in AKI biomarker literature (IV). The novel DCA, for example, enables risk model comparison, providing the net benefit of the new model compared to the old one at the chosen threshold probability [166,167]. A threshold probability weighs the false positives against the false negatives, an essential issue to be solved before implementing a new biomarker. Indeed, the evaluation of a biomarker's clinical usefulness involves numerous steps, and choosing appropriate statistical methods is mandatory [168].

Some study limitations must be addressed. First, these studies were *post hoc* analyses of the FINNAKI study or its substudies. Although the required sample size was calculated whenever possible, we could not influence the patient number in all the groups. Second, despite prompt plasma and urine sample collection upon ICU admission, a considerable number of patients already fulfilled AKI criteria at sampling time. This is a common problem in AKI biomarker studies and compromises calculations of the biomarkers' predictive values. Third, OD, UO, and fluid balance records may have been incomplete preceding ICU admission and study enrollment. Furthermore, up to one-third of the patients in our studies did not have baseline Cr measured, and we had to use back-calculation with the MDRD formula. AKI incidence was lower among these patients than among those having baseline Cr. Thus, by using

estimated baseline Cr values, we probably missed some AKI cases. This, also, is a universal drawback of AKI biomarker studies. Using the more sensitive CKD-EPI formula might have changed the picture. Fourth, we did not have daily patient weight data to assess fluid balance (I). Fifth, we did not normalize urine biomarker levels for urine Cr [238]. Sixth, considering Study II, the maximum SOFA score without the renal component was associated with AKI and thus plasma biomarker levels may have mirrored disease severity rather than AKI. Seventh, no major changes in ICU treatment protocols took place during the 6-month study enrollment period, but since then, the potential harms of excessive fluid loading have raised concern. This awareness has changed fluid therapy practices, which might have influenced Study I results had it been conducted today. Still, more restrictive fluid management protocols would probably further underline our results. Finally, the study participants were Caucasians from a high-income state with a public healthcare system. Applying the results to more ethnically diverse or otherwise different populations and health care systems must be done with caution.

Some of the definitions we applied have evolved since the planning of the FINNAKI study from which our data derived. Differences between AKIN and KDIGO AKI criteria are minor. The Sepsis-3 definition, on the other hand, is somewhat more sensitive than its predecessor [42], and, importantly, its clear OD definition is simpler for the clinician than the vague ACCP/SCCM criteria. We applied the Sepsis-3 criteria *post hoc* and found that nearly all the patients included using the former ACCP/SCCM sepsis criteria [37] fulfilled the Sepsis-3 criteria as well (III–IV). However, we do not know how many FINNAKI study participants not fulfilling the former criteria would have been included in our studies had the Sepsis-3 criteria been applied in the first place. We may have lost some patients from the less severe end of the sepsis syndrome.

Regarding fluid balance assessment (I), daily weighing should probably have been included in the CRF when planning the original study despite resources and equipment in ICUs varying. Fluid input and output calculations are only a rough estimate of the actual fluid balance. No consensus protocols for commencing RRT in the ICU exist, so the decision to start the treatment was made by the clinician. The reasons for these decisions were not recorded, only decisions to restrict care were. The fact that RRT indications and practices vary substantially in ICUs around the world must be considered when appraising biomarker studies and their generalizability.

Most importantly, we analyzed data from a prospective cohort study. The observational nature of the data and retrospectively designed substudies carry well-known limitations. An association does not equal causation. Even in the studies with reasonably large sample size, 484 (IV) to 2044 (I) patients, some outcome events were too rare and subgroups too small to draw conclusions.

6.7 Clinical implications and future aspects

Studying a large, non-selected ICU cohort, we demonstrated the futileness of adjusting Cr for fluid balance to diagnose AKI. Instead, we recommend applying the full KDIGO criteria, including UO, in clinical practice as well as in future trials. The current KDIGO AKI definition has been explicitly criticized for including Cr, a late marker of kidney function, not injury [239]. Definition weaknesses inevitably hamper AKI biomarker studies, and revision of the criteria is an important future target.

The present results support the experimentally observed inflammatory mechanisms of septic AKI. Besides the association of systemic inflammation with AKI, we found that high urine MPO, which could not be explained by plasma levels alone, was associated with septic AKI. This could indicate renal neutrophil infiltration and activation—similarly as seen in previous animal studies. Still, further studies are needed to confirm our results.

Understanding the kinetics of a biomarker is mandatory before its clinical implementation [133]. Our findings demonstrate that even small differences in sampling timing, in relation to the phase of sepsis, markedly influence the measured interleukin levels. Contrasting the rapid peaking and descending of IL-6 and IL-8 around OD onset, plasma HBP and MPO increased earlier and stayed elevated longer, making them more suitable for use as sepsis biomarkers. As another clinical implication, it has been suggested that heparin, an anticoagulant reducing HBP-mediated endothelial leak in an experimental setting [27], should be investigated in sepsis. The versatile anti-inflammatory properties of heparin have been in focus lately: low-molecular-weight heparin apparently benefits COVID-19 patients not only by anticoagulation but also by reducing IL-6 release and biological activity [240]. According to our findings on HBP kinetics, a sepsis treatment trial studying heparin should begin as soon as the patient enters the hospital. To achieve early diagnosis, pre-hospital alertness for sepsis symptoms is required and should be advanced with repeated campaigns for health care professionals as well as the general public.

Without effective prophylaxis and curative treatment for septic AKI, the present strategy has been early recognition of the impending OD and avoiding additional predisposing factors, as much as possible. Prognostication, that is, detecting AKI worsening or predicting the need for RRT has also raised interest. A better understanding of AKI progression could help in optimally directing limited critical care resources. The ongoing AKI biomarker search has not declared an indisputable winner, “renal troponin”, yet, but laboratory tests for some candidates are commercially available [241,242]. Conditional recommendations for the use of damage biomarkers together with clinical assessment and functional markers have been published [64], but as clinicians,

we are still far away from knowing how, when, and which biomarker(s) to use, or not use, in varying clinical situations [243]. Sepsis is a particular problem as inflammation is a confounding factor for many proposed AKI biomarkers [193], including NGAL. We scrutinized the added value of uNGAL as a predictive marker for AKI, RRT and 90-day mortality in sepsis patients. Our results indicate that uNGAL, although used in some countries, unfortunately does not have much incremental value for these purposes. Because clear biomarker cut-offs are difficult to define for the heterogenous ICU patient population, it has been proposed that serial measurements showing trends could improve the care of an individual patient. So far, no clinical randomized trial demonstrates the benefit of biomarkers to prevent clinically important adverse outcomes. A major obstacle to such septic AKI trials in the ICU is that, according to our data, many sepsis patients already present with AKI upon ICU admission—too late to be prevented by the intensivist.

Although statistically reasonable, collecting large representative sepsis cohorts for researcher-driven purposes may not be economically feasible. The recent COVID-19 pandemic may have a silver lining as the research burst on inflammatory mechanisms and immunomodulatory therapies may benefit sepsis patients as well. At least we can learn from the global, pragmatic, constantly evolving COVID-19 treatment trials how large cohorts can be collected with worldwide co-operation. Besides trials, we should improve automated data collection and utilization of electronic medical records and artificial intelligence. Additionally, increasing the use of databases created for quality improvement, benchmarking, and other applicable purposes is important. Based on these comprehensive albeit slightly lower quality data, randomized controlled trials could be targeted wisely.

Proteomics will probably help identify potential players involved in sepsis and AKI, but few candidates pass the cumbersome route to clinical usefulness. One reason for the slower than expected progress in septic AKI management might be the surprisingly scarce basic research on the subject. A review by Nakano revealed recently that only 1% of all AKI literature are basic science papers on septic AKI [244]. We should humbly head back towards the physiological grounds of renal dysfunction and recovery; not desperately seek biomarkers before understanding the mechanisms of septic AKI. The emerging evidence on neutrophil activation in septic kidneys requires further studies, preferably also at organ level. New technologies enabling leaping from cell cultures toward complete laboratory-grown organs create exciting visions for future research on the tissue-level mechanisms of septic OD.

7 CONCLUSIONS

Based on these studies, the following conclusions can be drawn:

1. Adjusting Cr for cumulative fluid balance in ICU patients did not change AKI incidence clinically meaningfully. We found no difference in 90-day mortality between patients reclassified in either direction when using the full set of KDIGO criteria with or without Cr adjustment.

2. Proinflammatory cytokines in plasma and urine were higher in patients with septic AKI compared to those without. Concomitant plasma and urine MPO measurements suggest renal neutrophil accumulation and activation. Neutrophil activation in plasma and urine was associated with septic AKI.

3. In evolving sepsis, intravascular neutrophil activation already occurred before the first observed OD and lasted longer than the initial proinflammatory cytokine peak.

4. Analyzing critically ill sepsis patients with novel statistical instruments, uNGAL did not add value to routine clinical and laboratory evaluation in prediction of AKI, RRT or 90-day mortality.

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Errata

Page 68, Table 12: Erroneus placement of asterisks (*) in the column titled "Severe AKI (KDIGO 2-3, n=134)". Corrected Table 12 presented below.

Table 12 Associations of variables explored in the univariable models with outcomes in Study IV. Modified from table in Törnblom et al. Annals of Intensive Care 2020;10:51, supplementary information, with permission from SpringerOpen (Creative Commons Attribution 4.0 International License).

	Data available (of 484)	**Two-sided p-values for outcomes**			
		AKI (KDIGO 1-3, n=217)	**Severe AKI (KDIGO 2-3, n=134)**	**RRT (n=46)**	**Death by day 90 (n=136)**
Age	484	0.022*	0.450*	0.160*	<0.001*
Gender (male)	484	0.636*	0.180*	0.339	0.119*
Diabetes	484	0.663*	0.016*	0.113*	0.204*
CKD	484	0.016*	0.023*	0.003*	0.009*
COPD	476	0.221*	0.241*	0.801	0.680
Liver disease	478	0.596*	1.000	1.000	<0.001*
Systolic heart failure	484	0.958*	0.404*	0.269	0.025*
Hypertension	484	0.605*	0.669*	0.590	0.236*
Atherosclerosis	484	0.195*	0.715	1.000	0.294*
ACE or ARB	477	0.295*	0.783	1.000	0.792
NSAID	466	0.279*	0.263*	0.611	0.547*
Corticosteroids	481	0.506*	0.859	0.856	0.001*
Pre-ICU hypovolemia	484	<0.001*	<0.001*	0.003*	0.598
Pre-ICU diuretics	484	0.040*	0.020*	0.700	0.184*
Pre-ICU colloids	484	0.109*	0.179*	0.422	0.737
Pre-ICU hypotension	484	<0.001*	<0.001*	0.013*	0.007*
Nonoperative admission	484	0.776*	0.407*	0.672	0.005*
Emergency surgery	482	0.769*	0.336*	0.200	0.024*
SAPS II (-age and renal points)	484	0.192*	0.491*	0.466	<0.001*
Highest lactate (day 1)	484	<0.001*	<0.001*	0.002*	<0.001*
Acute liver failure	484	0.143*	0.008*	0.163	0.002*

Variables with smallest p-values*, restricting the number of covariates to 1 per 8 dependent endpoints, were included in multivariate logistic regression analyses with corresponding endpoints.

ACE, angiotensin convertase enzyme-inhibitor (permanent medication); ARB, angiotensin II receptor blocker (permanent medication); CKD, chronic kidney disease; colloids, starch or gelatin; COPD, chronic obstructive pulmonary disease; ICU, intensive care unit; NSAID, non-steroid anti-inflammatory drug (permanent medication); SAPS II, Simplified Acute Physiology Score